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EXPRESSION AND LOCALIZATION OF MT1-MMP AND ITS ACTIVATING ENZYME FURIN IN THE GLOMERULAR WALL OF SHORT AND LONG TERM DIABETIC RATS

EXPRESSION ET LOCALISATION DE LA MT1-MMP ET DE LA FURINE DANS LA PAROI GLOMÉRULAIRE DE RATS DIABÉTIQUES

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EXPRESSION AND LOCALIZATION OF MT1-MMP AND ITS ACTIVATING ENZYME FURIN IN THE GLOMERULAR WALL OF SHORT AND LONG TERM DIABETIC RATS

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SUMMARY

Diabetic glomerulopathy has been traced to shifts in balance between the synthetic and degradative pathways of the glomerular basement membrane, a key player in the permselectivity of macromolecules. The goal of this study was to trace the expression and localization of MT1-MMP and its activating enzyme furin, key proteins involved in basement membrane turnover, in short- and long-term diabetic rat renal tissues.

To verify these assumptions, the expression of MT1-MMP and furin was evaluated in young and old, control and diabetic rat renal tissues. Immunoelectron microscopy revealed that the overall expression of MT1-MMP and furin is reduced in plasma membranes of cells of old normoglycemic animals, a phenomenon that is exacerbated in long-term diabetic animals. This observation supports the prevailing theory that diabetes fosters an acceleration in the aging process. Western blots were also performed on glomerular lysates. Interestingly, while biochemical results confirmed a decrease in MT1-MMP expression, an increase in furin was observed. Immunocytochemical studies resolved this discrepancy by tracing the increased furin expression in Golgi and ER membranes of podocytes, indicating that furin might be retained in the biosynthetic pathway in a diabetic environment. This suggests that while furin is overexpressed in diabetes, it is unable to reach the cell surface to contribute to extracellular matrix turnover.

Key words: diabetes, glomerulosclerosis, MT1-MMP, furin, GBM turnover

SOMMAIRE

Les glomérulopathies diabétiques sont caractérisées par un déséquilibre entre les voies de synthèse et de dégradation de la membrane basale glomérulaire (MBG), une structure essentielle à la permsélectivité des macromolécules. L'objectif était de suivre l'expression et la localisation de la MT1-MMP et de son enzyme d'activation la furine, deux protéines essentielles pour le renouvellement de la MBG, dans les reins de rats diabétiques.

Pour vérifier cela, l'expression de la MT1-MMP et de la furine a été évaluée dans les tissus rénaux de rats jeunes et âgés, contrôles et diabétiques. L'expression de la MT1-MMP et de la furine semble être réduite dans les membranes plasmiques des cellules glomérulaires des animaux âgés, mais par ailleurs normaux, un phénomène qui est aggravé chez les animaux diabétiques. Cette observation appuie la théorie selon laquelle le diabète accélère le processus normal de vieillissement. L'immunobuvardage sur des lysats glomérulaires a effectivement confirmé une baisse d'expression de la MT1-MMP. En revanche, en immunocytochimie, une hausse de niveau d'expression de la furine a été observée. Cette discordance a été résolue par immunocytochimie par la détection accrue de furine dans les membranes du Golgi et du réticulum endoplasmique. Cette observation suggère que la furine est retenue dans la voie biosynthétique des podocytes lors du diabète. Malgré sa sur-expression, la furine semble incapable d'atteindre la surface cellulaire pour contribuer au renouvellement de la MBG.

Mots clés: diabète, glomérulosclerose, MT1-MMP, furine, renouvellement de la MBG

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THESIS

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List of Symbols and Abbreviations

THESIS

α	Alpha
β	Beta
Å	Angström
AGE	Advanced glycation end products
CL	Capillary lumen
CTL	Control
GBM	Glomerular basement membrane
ECM	Extracellular matrix
END	Endothelium
HSPG	Heparan sulfate proteoglycan
kDa	kilo-Dalton
mRNA	Messenger ribonucleic acid
nm	Nanometer
P	Podocyte
R-H-R-R	Arginine-Lysine-Arginine-Arginine
STZ	Streptozotocin
TGF-β	Transforming growth factor beta
US	Urinary space

ARTICLE

%	Percent
°C	Degree Celsius
µg	Microgram
µm	Micrometer
CL	Capillary lumen
G	Golgi
GBM	Glomerular basement membrane
ECM	Extracellular matrix
EM	Electron microscope
ER	Endoplasmic reticulum
Fig	Figure
fur	Furin gene
kDa	kilo-Dalton
kg	kilogram
L	Liter
mg	Milligram
min	Minute
mol	Mole
mmol	Millimolars
MMP	Matrix metalloprotease
mRNA	Messenger ribonucleic acid

MT1-MMP	Membrane type-I matrix metalloprotease
nm	Nanometer
N-terminal	Amino terminal
P	Podocytes
pH	Hydrogen cation concentration
RER	Rough endoplasmic reticulum
RHRR	Arginine-Lysine-Arginine-Arginine
rpm	Rotations per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electropheresis
SEM	Standard error to the mean
TGF-β	Transforming growth factor beta
US	Urinary space

Foreword

Portions of the results of this thesis were presented at the following meetings :

BOUCHER, E., MAYER, G., BENDAYAN, M. **LOCALISATION ULTRASTRUCTURALE DE LA MT1-MMP DANS LA MEMBRANE BASALE GLOMÉRULAIRE DE RATS DIABÉTIQUES**. 22^{e} journée scientifique du Département de pathologie et biologie cellulaire, Montréal (Québec), Mai 2004.

BOUCHER, E., MAYER,G., BENDAYAN, M. **EXPRESSION OF GLOMERULAR MEMBRANE TYPE-I METALLOPROTEINASE IN RENAL TISSUE OF DIABETIC RATS.** 12th International Congress of Histochemistry and Cytochemistry, La Jolla (California), July 2004.

BOUCHER, E., MAYER, G., BENDAYAN, M. **LOCALISATION ULTRASTRUCTURALE DE LA MT1-MMP DANS LA MEMBRANE BASALE GLOMÉRULAIRE DE RATS DIABÉTIQUES**. Congrès annuel de l'Association Diabète Québec (ADQ), Québec (Québec), Novembre 2004.

BOUCHER, E., MAYER, G., BENDAYAN, M. **EXPRESSION ET LOCALISATION DE MT1-MMP ET FURINE DANS LA PAROI GLOMÉRULAIRE DE RATS DIABÉTIQUES.** 23^{e} journée scientifique du Département de pathologie et biologie cellulaire, Montréal (Québec), Juin 2005.

Dedications

À mes parents qui m'ont envoyé leur amour sure une brise tunisienne durant ces deux dernières années.

À Pascale et Marc-Antoine, les deux êtres les plus drôles sur cette terre.

Introduction

The human kidney is a veritable marvel of creation. There is no need to question the absolute indispensability of kidneys once we learn that the total volume of our circulating blood passes through the kidneys once every five minutes! This amounts to a rough total of seventy liters of blood plasma per day! The kidneys not only inspire us to cite staggering statistics that require exclamation points, but also perform the extraordinary and vital tasks of overseeing water and electrolyte homeostasis, regulating blood and extracellular fluid chemical composition, maintaining blood pressure through release of the renin hormone, stimulating red blood cell synthesis through release of erythroprotein, and simply being an all around great pair of organs (Leeson and Leeson, 1981).

Anatomically speaking, the individual kidney resembles a bean…or is it the bean that resembles a kidney? Regardless of any philosophical debate, the kidneys are located in the posterior part of the upper abdomen, and each kidney is about 10-12 centimeters in length and 2.5 centimeters thick (Leeson and Leeson, 1981). The three most prominent anatomical features seen in a transverse kidney section are the outer cortex, the inner medulla, and the pelvis, a hollow inner structure that joins with the ureters (Tisher and Madsen, 1986). In the medial sections of each kidney is an indentation called the hilum which serves as an exit point for the ureter and gives way to nerves, blood and lymphatic vessels entering and leaving (Leeson and Leeson, 1981). Other distinguishing features include the renal

capsule serving as a protective membrane, as well as right and left adrenal glands which, as the name implies, sit snugly on top of each kidney.

The Nephron

The interior medulla of each kidney is adorned with 8-18 pyramids that are striated in appearance. These are positioned with their tips, the renal papillae, facing towards the hilum and their bases aligned with the edge of the renal cortex (Leeson and Leeson, 1981). The renal cortex extends between each renal pyramid creating structures called renal columns. If we were to zoom into the medulla and cortex we would encounter a myriad of tiny microscopic convoluted structures called nephrons. At last, with the acquaintance of the nephron, we make the leap from structure to function and are closer to understanding the essence of the kidney. Also known as the functional units of the kidney, nephrons number slightly over one million in each kidney (Tisher and Madsen, 1986). Embedded in both the cortex and medulla, they are assigned the delicate task of filtering the blood plasma, that is, producing a filtrate while retaining the cellular elements of the blood and plasma proteins in circulation (Farquhar, 1991). The nephron may well exhibit a baffling hodge-podge of loops and twists and turns reminiscent of a Cirque du Soleil contortionist, but within this seemingly disorderly arrangement exists an highly organised and complex system of gradients through which alternating functions of secretions and reabsorptions result in the production of urine.

The essential components of the nephron are the glomerulus and Bowman's capsule, the proximal convoluted tubule, the ascending and descending limbs of the loop of Henle, and the distal convoluted tubule (Tisher and Madsen, 1986). The glomerulus and Bowman's capsule form the renal corpuscule which lies in the cortex while the renal tubule comprising of the thin limbs and connecting segment, the proximal and distal tubules, extends into the medullar pyramid (Leeson and Leeson, 1981). Given that we are interested in the production of the ultrafiltrate of blood plasma, the following information will focus solely on the renal glomerulus where this process takes place.

The Glomerulus

The glomerulus has been accurately yet perhaps unglamorously described as a tuft of capillaries. Within the layers of the Bowman's capsule, blood enters the glomerulus through the afferent arteriole and exits through the same opening by the efferent arteriole. The afferent arteriole branches into a capillary network where hydrostatic blood pressure forces the glomerular filtrate out into the capsular space to eventually be funneled into the renal tubule where reabsorption of most fluids and salts take place (Farquhar, 1991). A transversal cut of the glomerular capillary tuft unveils where ultrafiltration takes place. The different structures observed, collectively called the glomerular wall, impose a precise permselectivity size and charge barrier to the blood plasma and will be extensively reviewed in the following paragraphs.

The Glomerular Wall

In a remarkable feat of precision, the glomerular capillary wall allows water and small solutes to pass readily into Bowman's space while rejecting albumin and other large proteins with great efficiency. In its entirety, this meticulous engine of ultrafiltration is composed of three noteworthy parts- endothelial cells, extracellular basement membrane, and epithelial cells **(Figure 1)**.

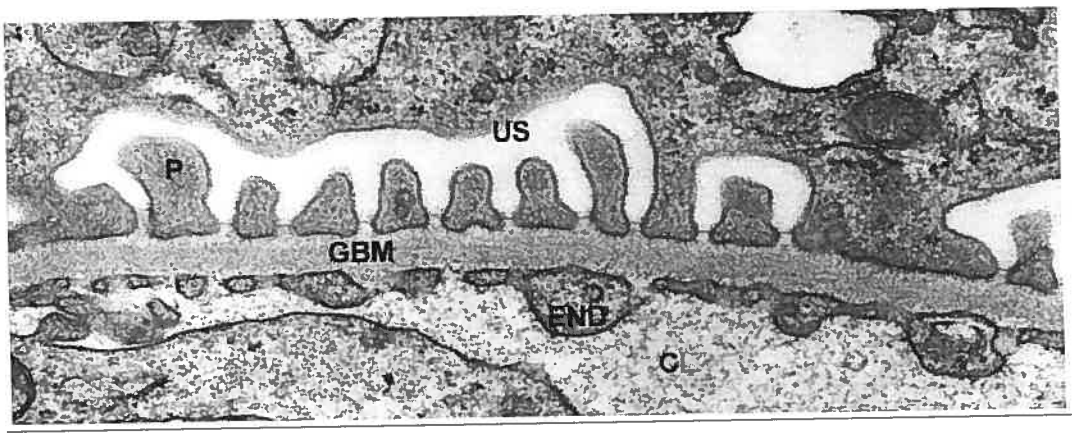

FIGURE 1: The glomerular wall. Electron micrograph of the glomerular wall of tissues fixed in osmium and embedded in Epon. Endothelium (END) and podocytes (P) flank the glomerular basement membrane (GBM) which together make up the filtration barrier to the blood plasma. The filtrate moves from the capillary lumen (CL) and into the urinary space (US) by way of the slit diaphragms at the junction of GBM and podocytes. X40 000 (Courtesy of I. Londoño)

Endothelium

The endothelium of glomerular blood capillary vessels represents the first physical barrier in blood filtration. The most salient feature of this endothelial layer is the presence of fenestrae that are relatively large in size (50-100 nm) and are open, that is, lack the usual diaphragms that are present in other fenestrated capillaries (Farquhar, 1991). While it may seem like these large open windows offer little or no resistance to the passage of blood plasma, recent evidence emphatically argues that heavily fenestrated endothelial cells are important elements of the highly permselective glomerular barrier (Haraldsson and Sörensson, 2004). Admittedly, the actual fenestrae are too large to effectively sieve macromolecules (Deen, 2004), but the cell coat or glycocalyx secreted by endothelial cells themselves has been under recent scrutiny for its potential size- and charge-selective properties (Haraldsson and Sörensson, 2004). To date it has been established that this layer coating endothelial cell surfaces is made up of glycosaminoglycans and proteoglycans in combination with plasma proteins, but further investigations on its composition and turnover rate are needed before defining its precise role in glomerular permselectivity (Haraldsson and Sörensson, 2004). One well-established fact, however, is the presence of the negatively-charged protein podocalyxin at the cell surface of endothelial cells (Dekan et al., 1991; Kerjaschki et al., 1984; Latta et al., 1975). Podocalyxin reportedly pushes away other negatively charged molecules such as albumin. The fact that endothelial fenestrae are open and larger than those of other fenestrated capillaries makes them highly efficient for filtration, but this also renders the GBM

vulnerable to toxic injury such as that caused by hyperglycemia in the blood, a phenomenon we will explore in detail shortly.

Basement Membranes

The earliest histological descriptions of basement membranes date back to 1857 from the laboratories of Todd and Bowman (Kefalides, 1973). In broad terms, basement membranes are recognized as extracellular matrices associated with the epithelial and endothelial linings of almost all organs of the body. They are found wherever cells (other than connective tissue cells) meet connective tissue (Farquhar, 1991). A century after having identified these characteristics, great leaps in studying tissue morphology were made with the invention of the electron microscope and the ultrastructure of the basement membrane was described (Kefalides, 1973). Visualization at unprecedentedly high magnifications unveiled the *lamina densa*, 20-50 nm in thickness, running parallel to the basal cell membranes of either epithelium or endothelium, and separated from these by the lamina lucida, a lighter, 10 nm layer (Farquhar, 1991). Histochemical studies in the 1960s revealed the collagenous and noncollagenous glycoprotein and proteoglycan content of basement membranes with various chemical staining experiments. For example, Kefalides and Denduchis (1969) used pronase on canine basement membranes to demonstrate for the first time the overwhelming presence of collagen. Before that, Leblond (1957) indicated the presence of carbohydrates with the periodic anti-Schiff reagent. Further biochemical investigations throughout the sixties by several research groups characterised the

complete amino acid and carbohydrate composition of basement membranes, underlining their high content of hydroxyproline, glycine, and hydroxylysine as well as glucose, galactose, fucose and mannose (Kefalides, 1973). The general concensus is that components of basement membranes are secreted by cells lying adjacent and do not differ significantly in content from organ to organ but rather in quantity and structural organization, reflecting tissue function and specificity (Kefalides and Denduchis, 1969). The participation of basement membranes in processes such as tissue repair and regeneration, as well as cellular attachment, migration and development, has provided important insights into research on cancer and diabetes (Erickson and Couchman, 2000; Timpl and Brown, 1996).

The Glomerular Basement Membrane

An extensively studied and long-time favorite of basement membrane-aficionados is the highly specialized basement membrane of the glomerular capillaries of mammalian kidneys. Pioneering the ultrastructural description of the glomerular basement membrane under electron microscopy were Rhodin (1955), Yamada (1955), Kurtz and McManus (1959), and Farquhar (1960) among others. Their ground-breaking studies paved the way for more involved histochemical and biochemical analyses of the glomerular basement membrane, analyses that eventually ascribed it its two main functions of support and selective filtration (Kefalides, 1973).

The glomerular basement membrane bears certain distinguishing features that sets it apart from other basement membranes. It is thicker (350 nm in humans) and more compact, it is formed by fusion of endothelial and epithelial basement membranes, and it faces endothelial and epithelial cell layers on both surfaces (Farquhar, 1991; Desjardins and Bendayan, 1991). The particular structure-function relationship of the glomerular basement membrane in relation to other extracellular matrices was well defined. Desjardins et al. demonstrated that the specific spatial distributions of the main components of the GBM were instrumental in determining its filtration properties. This was done by showing how the passage of albumin is increasingly restricted to the subendothelial layer as maturation of the GBM progresses (Desjardins and Bendayan, 1991; Bendayan et al., 1986). It has thus become dogma that the structural and chemical composition of the mature glomerular basement membrane is what renders it its unique filtration properties.

Throughout the 1970s, the works of Spiro and Kefalides with purified GBM fractions provided a great wealth of information on the fine architecture of the glomerular basement membrane (Farquhar, 1991). The 1980s ushered in the era of immunolabeling in electron microscopy which also proved to be a valuable tool in elucidating not only the content but also the distribution of GBM constituents (Courtoy et al., 1982; Stow et al., 1985; Kerjaschki et al., 1986). This was done through the purification of proteins from renal glomeruli and tubules which were then used as antigens to prepare specific antibodies (Kerjaschki et al., 1986). The

culmination of this work, mostly carried out in Farquhar's laboratory, revealed that the major components of the GBM are collagen type IV, laminin, heparan sulfate proteoglycan, nidogen or entactin, and BM40 (Farquhar, 1991). Electron microscopy uncovered specific patterns of distribution of collagenous components, proteoglycans and glycoproteins in laminae rarae and laminae densae, eventually leading to sophisticated three-dimensional models of glomerular basement membrane architecture. The sturdy scaffolding networks created from the binding affinities existing between type-IV collagen, heparan sulfate, laminin, nidogen and entactin underlines the basement membrane's tough supportive qualities (Farquhar, 1991).

The highly selective filtering properties of the GBM are dependent on the quality, the quantity, and the distribution of its constituents. Increasingly sophisticated physiological blood plasma clearance studies have demonstrated that the glomerular basement membrane serves primarily as a charge- and size-selective barrier (Farquhar, 1991). For example, the presence of highly negatively charged regions of glycosaminoglycans containing heparan sulfate impose a significant charge barrier (Farquhar, 1991), conferring them the role of the main anionic site for GBM charge selectivity (Akthar, 2004). It has been speculated that the size-selective sieving properties of the GBM are a property of the highly compact meshwork of type-IV collagen (Kanwar, 1984). Collagen is composed of three alpha-chains forming triple-helical molecules, which form a tightly cross-linked network structure. It is chiefly concentrated in the *lamina densa* of the GBM

(Kanwar, 1984). Although the exact size of the pores formed by networks of collagen and other GBM proteins have not been precisely determined, it is widely accepted that size restriction is an inherent quality of the highly specialized meshwork of the GBM. Morphological approaches using the large (60 Å) ferritin molecule as a tracer revealed that much of the size-selection occurs at the level of the *lamina rara interna* (Farquhar et al., 1961) while smaller molecules like horseradish peroxidase can traverse the membrane (Kefalides, 1973). The significance of the GBM as an important size-barrier was also highlighted by Farquhar in an experiment involving dextran tracer molecules (Farquhar, 1975), a finding which actually shifted the attention of the primary filtration barrier from podocytes to the GBM. Albumin has also played a central role in identifying the specific size retention areas in the glomerular basement membrane (Bendayan et al., 1986; Londoño et al., 2003). Immunocytochemistry revealed the presence of albumin at the level of the endothelial cell basal plasma membrane and on the subendothelial side of the *lamina densa* of the GBM, indicating that albumin retention occurs at these sites (Bendayan et al., 1986). Following up on these experiments, it was shown that glycated albumin, once in circulation, penetrates the glomerular wall of normal animals deeper than nonglycated albumin and eventually reaches the urinary space. This provided evidence that albumin normally does not pass through the glomerular wall while an abnormal glycated version of the protein is able to modify the glomerular filtration properties in such a way as to be able to reach the urinary space (Londoño et al., 2003).

Podocytes

The epithelial cells of the glomerular wall are called podocytes which separate the endothelial capillary vessels from the urinary space of the Bowman's capsule. In scanning electron microscopy it is possible to visualize their unique cell body prolongations which resemble interlacing fingers covering the entire surface area of capillary vessels (Guyton and Hall, 2000; Tisher and Madsen, 1986). From these elongations emerge smaller secondary projections called pedicels. Pedicels interdigitate with other pedicels from neighbouring podocytes and firmly anchor themselves in the *lamina rara externa* of the GBM (Tisher and Madsen, 1986; Pihlajaniemi, 1996). It is interesting to note that adjacent pedicels are most likely projections from different podocytic cell bodies (Tisher and Madsen, 1986).

The podocytes are the most voluminous cells in the glomerulus (Guyton and Hall, 2000). Along with a well-developed Golgi, they contain free ribosomes as well as rough and smooth ER cisternae (Tisher and Madsen, 1986), which indicates their marked propensity for protein synthesis. Kurtz and Feldman (1962) pointed out that podocytes are largely responsible for the biosynthesis of the glomerular basement membrane. In fact, it has been widely documented that enzymes responsible not only for GBM synthesis but also turnover and maintenance, are largely synthesized within podocytic cell bodies (Lee et al., 1993; McCarthy, 1997). Of these enzymes, the family of matrix metalloproteases play a crucial role as will be seen shortly.

In the past decade, attention has been shifted to podocytes for a clearer understanding of permselectivity. The spaces existing between pedicel interdigitations vary between 25 and 60 nm and are called filtration slits (Pihlajaniemi, 1996), while the porous thin membranes that traverse the filtration slits are called slit diaphragms (Leeson and Leeson, 1981). The slit diaphragms of adjacent pedicels are linked to each other by specialized plasma membrane adherens junctions (Guyton and Hall, 2000; Tisher and Madsen, 1986), and the integrity of this arrangement is largely dictated by the presence of a sialic acid-rich glycocalyx (Farquhar, 1991).

It was long believed that the primary filtration barrier of the glomerular wall resided in the filtration slit regions of podocytic interdigitations. Indeed, this area was shown to exhibit size-restrictive properties (Rodewald and Karnovsky, 1974), and is also essential for glomerular wall reinforcement in the face of high hydrostatic blood plasma pressures (Kerjaschki, 2001). It is only with the recent molecular era that our understanding of podocytes and their role in filtration has greatly advanced. Kerjaschki greatly facilitated our comprehension of the increasingly complex molecular characteristics of the podocytes by separating its plasma membrane area in three distinct microdomains: the slit diaphragms at the junction of adjacent podocytes, the basal membrane which touches the GBM, and the apical membrane.

Slit Diaphragm

The most influential study of the fine structure of the slit diaphragm was provided by Rodewald and Karnovsky back in 1974 (Rodewald and Karnovsky, 1974). They interpreted electron microscopy images of slit diaphragms as zipper-like structures that are arranged in such a way to restrict passage of molecules. The zipper resembles a central dense region with openings roughly the size of albumin (Deen, 2000). The novel application of electron tomography was able to show an increased complexity of the pores, more tortuous and irregular than previously supposed (Deen, 2000). Characterization of the molecular composition of slit diaphragms boomed in recent years with the identification of nephrin (Holzman et al., 1999; Kerjaschki, 2001), NEPH-1 (Kerjaschki, 2001), CD2AP (Kerjaschki, 2001; Li et al., 2000), and podocin (Kerjaschki, 2001; Schwarz et al., 2001). The newly discovered protein that received the most attention was the transmembranous nephrin, found to be an adhesion protein necessary for the formation of junctions (Holzman et al., 1999; Kerjaschki, 2001). It was recently demonstrated that the fibers constituting the slit diaphragm appear to be formed largely by the association of extracellular strands of nephrin (Deen, 2000). The exact mechanism by which nephrin controls permselectivity is not yet clear but the Finnish congenital nephrotic syndrome emphasized the importance of nephrin which is absent in this disease (Akthar, 2004). Indeed, in nephrin-knockout mice, ordered slit diaphragm structures are no longer evident, and proteinuria results (Deen, 2000). Increasing evidence only further confirms that disruption of the slit

diaphragms is sufficient to cause abnormal loss of proteins in the urinary space (Hamano et al., 2002).

Basal Membrane

The basal plasma membrane of podocytes is anchored to the GBM through adhesion proteins, most notably integrin $\alpha 3\beta 1$ (Kerjaschki et al., 1989). $\alpha 3\beta 1$ bridges podocytes and GBM through association with paxillin, talin and vinculin on the cytoplasmic podocytic side (Drenckhahn and Francke, 1988), and collagen, fibronectin, laminin and entactin on the GBM extracellular matrix side (Kretzler, 2002). Another noteworthy constituent of the podocyte basal membrane is dystroglycan which is believed to regulate the position and spacing of proteins in the extracellular matrix. This would confer it a role in porosity and GBM permeability. Indeed, a fall in dystroglycan expression has been shown to coincide with podocyte effacement and proteinuria (Kerjaschki, 2001).

Apical Membrane

The main role of the apical membrane region of podocytes seems to be in the maintenance of structure and cohesion of podocytes (Kerjaschki, 2001). This function has been linked to the presence of podocalyxin, a highly negatively charged protein also present on the endothelium, as was mentioned earlier. This glycoprotein was found to be abundant on the apical surface of podocytes by Sawada et al. in 1986 who also discovered that neutralizing the charges on podocalyxin leads to alterations in podocytic processes and slit diaphragm

organisation, a finding which underlines the importance of its presence in podocyte maturation. Recent evidence also indicates that podocalyxin can also link itself to cytoskeletal actin by virtue of NHERV-2 and ezrin (Kerjaschki, 2001), which confers to podocyte projections their particular "feet"-like architecture.

Together, the endothelium, glomerular basement membrane and podocytes interact to form an highly intricate sieve designed to select molecules on the basis of size, shape and charge. Despite the comprehensive and compelling evidence offered on the structural and biochemical composition of the glomerular wall, the specifics of ultrafiltration still remain nebulous. Initially it was widely believed that the primary filtration barrier was located exclusively at the interdigitations of podocytes (Karnovsky and Ainsworth, 1972) but Bendayan and Farquhar produced persuasive evidence highlighting the size-restrictive properties of the glomerular basement membrane (Farquhar, 1975; Bendayan et al., 1986). Recently, more weight has been given to the epithelial slit diaphragms due to important advances in molecular biology and the identification of nephrin. A comprehensive study has shown how decreased expression of nephrin correlates with a loss in glomerular filtration integrity (Hamano et al., 2002). Finally, in 2004, Sörensson and Haraldsson argued that too much attention has been given to the GBM and epithelial podocytes in particular, while the endothelium probably has a much greater role in glomerular permselectivity than we believe. This is in light of new information concerning the cell surface coat covering endothelial

cells called the glycocalyx, and the identification of proteins within it that seem to be of vital importance for capillar permeability (Sörensson and Haraldsson, 2004). The debate rages on, yet one thing remains certain: the proper functioning of the glomerural wall is a function of the integrity, interdependence, and proper communication of *all* its parts.

Diabetic nephropathy

Whether we like it or not, our understanding of how something works is greatly advanced when that thing doesn't work. Such is the case with the glomerular filtration apparatus; much of our knowledge of its function comes from observation of pathophysiological situations which surround glomerulosclerosis. Malfunctioning as a result of diabetes leads to generalized proteinuria or abnormal loss of proteins into the urinary space due to an imbalance in the size and charge-selective barrier properties of the glomerular wall. The relevance of diabetes mellitus in today's society cannot be overemphasized: there is a 2-5% increase in the incidence of type-I diabetes but the real concern is the global increase in type-II, largely due to childhood obesity (Silink, 2002). Diabetic nephropathy, a condition that develops after exposures of tissues to chronic hyperglycemia, is one of the leading causes of renal failure in Western countries (Mason and Wahab, 2003). Diabetic patients account for nearly half of all patients on haemodialysis, the kidneys being home to the gravest pathophysiological consequences of this disease (Del Prete et al., 1998). The complications arising from these lesions lead to glomerular fibrosis which

progressively destroys the renal filtration unit and may eventually cause renal failure (Del Prete et al., 1998). Our project deals specifically with changes, structural and compositional, in the glomerular wall at the molecular level that accompany the onset of this devastating disease.

As early as 1959, morphological changes of the glomerulus in diabetic conditions were observed by electron and light microscopy (Farquhar et al., 1959). Studies by several different groups concurred that the most striking feature of a glomerular wall exposed to an hyperglycemic environment is the significant thickening of the glomerular basement membrane **(Figure 2)** (Kimmelstiel et al., 1962; Bloodworth, 1963; Østerby and Lundbaeck, 1970). Further investigations into this morphological discrepancy revealed an important correlation between GBM thickening and biochemical changes in the amino acid and sugar composition of the basement membrane (Spiro and Spiro, 1968; Beisswenger and Spiro, 1970; 1973; Beisswenger, 1976; Spiro, 1976). Pioneering work concerning the effect of diabetes on the biochemical composition of the basement membrane was thus undertaken. Kanwar demonstrated by autoradiography and biochemical analysis that proteoglycan synthesis decreased by 40% in diabetic rats relative to normal rats (Kanwar et al., 1983). In an elegant experiment employing glucosyltransferase, an enzyme involved in the synthesis of certain GBM components, Spiro and Spiro demonstrated increased levels of this signaling protein in diabetic kidneys relative to normal ones (Spiro and Spiro, 1971). Indicating that increased basement membrane synthesis in diabetes might be a

result of the overexpression of this protein, they also noted that this process is reversible through insulin administration. Additional studies signaled the effect of diabetes on the metabolism of the GBM, as well as the significantly reduced level of heparan sulfate, the aforementioned major player enforcing charge selectivity, in the GBM under diabetic conditions (Parthasarathy and Spiro, 1982; Brownlee and Spiro, 1979). Employing ultrastructural analyses, other studies also confirmed a decrease in heparan sulfate immunolabeling in the *lamina rara interna* of the GBM in diabetic rats (Desjardins and Bendayan, 1990; Vernier et al, 1992).

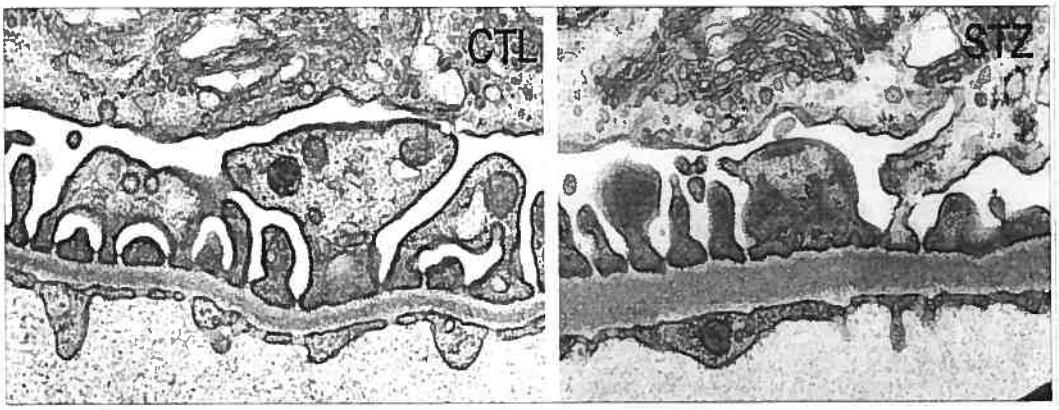

FIGURE 2: Comparison between glomerular walls of normal and diabetic animals. Electron micrograph of glomerular walls of rat renal tissues, 2-month old control (CTL) and 6-month old diabetic (STZ) animals. Significant thickening is observed in the glomerular basement membrane of the diabetic animal due to increased deposition of extracellular matrix components. X60 000 (Courtesy of I. Londoño)

While Spiro focused on biochemical modifications, the works of Østerby for the past three decades were instrumental in elucidating those structural changes that characterise glomerulopathy (Østerby and Gundersen, 1980; Gundersen and

Østerby, 1977; Østerby et al., 1993; Østerby et al., 1987; Østerby et al., 1988). Using streptozotocin-induced diabetes in rats, it was shown that generalized glomerular enlargement was apparent only four days after injection (Østerby and Gundersen, 1980). Progressive expansion of the mesangial matrix, and thickening of the glomerular and tubular basement membranes thus became universal hallmarks of human and experimental diabetic nephropathy (Del Prete et al., 1998). These findings provided the firm basis upon which countless experiments were inspired to supply additional information on the characteristics of diabetic nephropathy.

The technique of high-resolution immunocytochemistry using electron microscopy and immuno-biochemical quantifications offered great insights into the expression and distribution of structural proteins in the GBM. In diabetic patients with only mild glomerulosclerosis, increases in collagens type-IV, $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 4$, laminin, and fibronectin were reported while levels of perlecan decreased (Kim et al., 1991; Mohan et al., 1990; Shimohura and Spiro, 1987). Other factors found to be abundant in glomerular diabetic tissues were advanced glycated end products (Gugliucci and Bendayan, 1996; Bendayan, 1998). The main culprit of basement membrane expansion, however, is the high deposition of collagen. Increases in collagen deposition in GBM and mesangial matrix (Regoli et al., 1998; Nerlich et al., 1994) as well as an augmentation in collagen mRNA (Park et al., 1997; Fukui et al., 1992) have been well documented in advanced diabetic tissues. Desjardins and Bendayan brought to our attention the

modification in the spatial distribution of collagen type-IV in long-term diabetic animals (Desjardins and Bendayan, 1990). Normally concentrated in the *lamina densa*, collagen type-IV was found to be relocalized to the subendothelial side of the GBM in diabetic animals (Bendayan, 1985; Desjardins and Bendayan, 1990). No changes were observed in the distributions of laminin, entactin and heparan sulfate in those same animals (Desjardins and Bendayan, 1990). It thus became apparent that any modifications concerning the expression and distribution of structural proteins in the GBM were reflected in the three-dimensional scaffolding networks. Indeed, through immunocytochemistry in electron microscopy, it was possible to observe an alteration at the level of structural organisation of GBM components during the course of diabetes. Specifically, the endothelial side of the GBM gave rise to dense bundles of fibrils identified as basotubules which were found to be largely associated with type-IV collagen in diabetic animals (Inoue and Bendayan, 1995).

Our project focuses on specific factors leading to the occurrence of the most prominent morphological feature of glomerulopathy: the thickening of the glomerular basement membrane, a phenomenon which has been associated with a decrease in filtration area, abnormal loss of proteins in the urinary space and ultimately end-stage renal failure. With this important observation, it became apparent that perpetual maintenance and turnover of the molecules that make up the glomerular basement membrane is sine qua none for proper permselectivity. It is now widely accepted that the amount and composition of the GBM

extracellular matrix relies on the delicate interactions existing between synthetic and degradative pathways, and that various forms of glomerular diseases are characterized by shifts in this balance.

Membrane Type-I Metalloprotease

Nowadays, when someone says "tissue remodeling", the matrix metalloproteases (MMPs) immediately come to mind (well, among us molecular biologists anyway). This growing family of zinc-dependent metalloendopeptidases is the object of many studies dealing with extracellular matrix turnover. This is due to their prominent role as cleavers of practically all components of the extracellular matrix such as collagen, proteoglycans, fibronectin, and laminin (Bode et al., 1999). MMPs have thus been shown to be key players in processes involving degradation of pericellular proteins, thereby assuming pivotal roles in normal and pathological processes (Sternlicht and Werb, 2001).

Historically, MMPs were classified as collagenases, gelatinases, stromelysins, and matrilysins based on their cleaving specificities, but the growing number of MMP substrates has prompted us to group them according to structure (Egeblad and Werb, 2002). The 21 MMPs are divided into eight structural classes; five secreted and three membrane-type MMPs (Egeblad and Werb, 2002). The degradative functions of metalloproteases are largely kept in check by their endogenous tissue inhibitors (TIMPs) and disruption of this balance has been documented to result in serious diseases such as arthritis, tumor growth and

metastasis (Bode et al., 1999). Thus MMPs are able to regulate many biological processes and are closely regulated themselves.

MMPs share several structural and functional properties: they are synthesized as inactive zymogens containing a secretory signal sequence, a propeptide hinge, as well as hemopexin-like and catalytic zinc-binding domains (Lenz et al., 2000; Stamenkovic, 2003). Activation requires proteolytic removal of the propeptide prodomain (Egeblad and Werb, 2002). Of the currently known MMPs, MMP-1, MMP-13, MMP-3, MMP-2, MMP-9, and MT1-MMP all share the characteristic of being collagenases and thus have been extensively studied in the glomerulus (Lenz et al., 2000). In this project, we chose to study the membrane type-I matrix metalloprotease (MT1-MMP) due to its unique properties conferred by its location at the cellular membrane.

The membrane type-I metalloprotease distinguishes itself as a membrane-bound enzyme capable of exerting its cleavage activities from the vantage point of the cell surface (Bode et al., 1999). MT1-MMP possesses an additional stretch of around 20 hydrophobic amino constituting a transmembrane domain, followed by a short cytoplasmic tail **(Figure 3)** (Sato et al., 1994). Active MT1-MMP inserts itself into the plasma membrane with the catalytic domain facing the extracellular space, where it can cleave substrates in the extracellular matrix (Osenkowski et al., 2004). Localization of MT1-MMP at the cell membrane is perfect for pericellular proteolysis allowing for a new set of substrate targets, distinctive

interactions with TIMPs and a non-conventional mechanism of regulation involving enzyme internalization, processing and ectodomain shedding (Toth et al., 2002; Osenkowski et al., 2004).

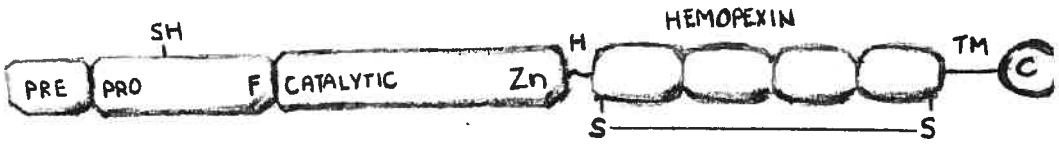

FIGURE 3: Domain structure of MT1-MMP. Signal sequence (PRE), propeptide (PRO) with a free zinc-ligating thiol (SH) group, furin-susceptible site (F), zinc-binding site (Zn), hinge region (H), transmembrane region (TM), and cytoplasmic tail (C). The hemopexin domain contains four repeats, the first and last are linked by a disulfide bond. (Illustration not to scale by Emmanuelle Boucher, adapted from Sternlicht and Werb, 2001: How matrix metalloproteinases regulate cell behavior. *Ann Rev Cell Dev Biol* 17: 463-516)

Current evidence indicates that MT1-MMP is able to regulate matrix turnover due to its ability to degrade matrix-associated molecules, either directly or via activation of downstream MMPs (Yana and Weiss, 2000). The dual role of MT1-MMP as activator of other MMPs capable of initiating activation of zymogen cascades, and as direct cleaver of ECM components, only underscores its vital function as an extracellular matrix remodeler and singles it out as a potential therapeutic target (Lenz et al., 2000; Ohuchi et al., 1997). The importance of MT1-MMP's role as a collagenase was demonstrated in mice deficient in MT1-MMP which suffered severe complications in remodeling of skeletal connective tissues, resulting in earlier death (Holmbeck et al., 1999). It has been speculated that MT1-MMP's strategic location confers it an advantage over other

collagenases in that it can accomplish localized and efficient collagen degradation at the cell surface (Hotary et al., 2000), as well as initiate zymogen activation cascades (Toth et al., 2003). One such cascade is the activation of pro-MMP-2 which was actually the mechanism by which MT1-MMP was first identified (Overall and Lopez-Otin, 2002). Here, active MT1-MMP binds TIMP-2 which generates a surface receptor for pro-MMP-2 (Osenkowski et al., 2004). The resulting MT1-MMP/TIMP-2/pro-MMP-2 ternary complex presents the bound pro-MMP-2 to a neighbouring TIMP-2-free MT1-MMP which will initiate the activation of pro-MMP-2. Once proteolytically processed by MT1-MMP, active MMP-2 is released into the extracellular space where it can carry out its cleavage activities (Bernardo and Fridman, 2003). This effectively changed our traditional view of TIMPs as inhibitors to that of versatile molecules capable of acting in the promotion of pericellular proteolysis. Indeed, MT1-MMP plays a dual role in the pathophysiological digestion of extracellular matrix through direct cleavage of the substrates and the indirect activation of pro-MMP-2 as well as other zymogen cascades (Cowell et al., 1998; Toth et al., 2003). MT1-MMP's wide substrate range specificity includes collagen types I, II and III (into typical ¾ and ¼ length fragments), laminin types 1 and 5, vitronectin, gelatin, proteoglycan fibrin, and aggrecan (Ohuchi et al., 1997). It is thus evident that appropriate expression and activity of this endoprotease is a mainstay for basement membrane maintenance and turnover.

Furin

As mentionned earlier, cleavage of the propeptide region is essential for MT1-MMP activation and subsequent proteolytic activity. Yana and Weiss demonstrated the existence of a proprotein convertase-MT1-MMP axis allowing for the unmasking of MT1-MMP's catalytic domain and conversion into a catalytically active species (Yana and Weiss, 2000). This was done through the identification of the RX(K/R)R basic motif in the propeptide region of MT1-MMP, a sequence of amino acids that can potentially be recognized by the proprotein convertase family of subtilisin-like proteases. Indeed *in vitro* studies using MT1-MMP deletion mutants confirmed that furin is capable of cleaving the proMT1-MMP zymogen (Yana and Weiss, 2000). It is thus apparent that cooperative interactions between proprotein convertases and membrane-anchored MMP's play an important role in regulating the remodeling of ECM.

Furin is a calcium-dependent endoprotease involved in the proteolytic activation of a large variety of proprotein substrates in secretory pathway compartments (Thomas, 2002). The importance of furin as a proprotein convertase capable of processing a wide range of precursors cannot be overemphasized as it has been shown to be ubiquitous in most cell types (Blanchette et al., 1997). Also, knockout of furin gene is embryonic lethal (Roebreck et al., 1998), underlining its importance in the maturation, function and activation of several hormones, growth factors, and cell surface receptors (Molloy et al., 1999). Specific amino acid motifs identified in furin's cytoplasmic tail have shown to target furin to different

cellular compartments; a finding which has shed light on furin trafficking between the trans-Golgi network, endosomal compartments and the cell surface (Plaimauer et al., 2001; Molloy et al., 1994; Mayer et al., 2003).

The physiological role of furin in glomerular cells was well characterized by Mayer et al.. Double immunogold labelings revealed that furin was co-localized along with pro-MT1-MMP in the biosynthetic pathway, and was also observed beyond the trans-Golgi network and on the cell surface of podocytes (Mayer et al., 2003). Mayer et al. propose an MT1-MMP/furin activation axis at podocytic and endothelial abluminal membranes facing the GBM for a type of focalized pericellular proteolysis essential for GBM turnover (Mayer et al., 2003). These novel findings on the cellular biology of podocytes prompted us to investigate whether the expression of these proteins was altered in a hyperglycemic environment. This would shed some light on the elements that characterize the integrated functions of cellular and extracellular elements involved in tissue-remodeling, and their disregulation in diabetes.

Objective of Study

The aim of our project is to characterize some specific molecular changes in the glomerular wall that accompany diabetic glomerulosclerosis. The possibility that the expression and/or localization of proteins involved in extracellular matrix turnover is modified is highly suggested by the thickening of the glomerular basement membrane. This is why the membrane type-I matrix metalloprotease, one such endoprotease involved in the cleaving of many ECM components, and its activating enzyme furin, were elected for study. It was decided that valuable comparisons could be made between short- and long-term diabetic animals and their age-matched controls. Rats were thus injected with streptozotocin and sacrificed after 2 and 12 months of maintaining a hyperglycemic state. Comparitive evaluations could then be undertaken at a morphological and biochemical level.

Immunocytochemical techniques with colloidal gold immunolabeling offer distinct advantages in evaluating these enzymes' expression and localization in the renal tissues of normal and diabetic animals. Antigenic sites of MT1-MMP and furin can be detected with their corresponding polyclonal antibodies which in turn can be revealed by the protein-A colloidal gold complex (Bendayan, 1995). This high resolution technique not only allows us to view the localizations of MT1-MMP and furin, but also to quantify their intensity in labeling.

Morphometrical analyses were greatly facilitated by virtual dissection of the glomerular wall into distinctive structures **(Figure 4)**. It is thus possible to quantitatively evaluate selected portions of glomerular cell surfaces suspected to harbor these matrix-remodeling enzymes.

FIGURE 4: Dissected areas of the glomerular wall for morphometrical evaluation. The plasma membrane surfaces of the glomerular wall were virtually separated into five distinct regions to facilitate morphometrical analysis: the abluminal (red) and luminal (green) podocyte membranes, the slit diaphragm (blue), and the abluminal (yellow) and luminal (pink) endothelial membranes. Gold particles per micrometer were measured. X40 000 (Courtesy of I. Londoño)

To further substantiate immunocytochemical data, we perform western blots on glomerular lysates, once again using polyclonal antibodies against MT1-MMP and furin. When discrepancies were observed between biochemical and

morphological results, immunocytochemistry once again proved to be an invaluable tool in resolving these inconsistencies by revealing changes in localization of the studied enzymes within the glomerular wall. The western blotting and immunogold techniques thus complement each other well, rendering it possible to determine overall expression and specific placement of proteins in the glomerular wall.

Article

EXPRESSION AND LOCALIZATION OF MT1-MMP AND ITS ACTIVATING ENZYME FURIN IN THE GLOMERULAR WALL OF SHORT AND LONG TERM DIABETIC RATS

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ABSTRACT

Diabetic glomerulopathy has been linked to shifts in balance between the synthetic and degradative pathways of the glomerular basement membrane, a key player in the permselectivity of macromolecules. The goal of this study was to trace the expression and localization of MT1-MMP and its activating enzyme furin, key proteins involved in basement membrane turnover, in short and long-term diabetic rat renal tissues.

Quantitative immunogold was carried out for MT1-MMP and furin and their expression was evaluated in renal tissues of young and old, control and diabetic rats. To corroborate immunocytochemical findings, western blots were performed on glomerular lysates.

Electron microscopy revealed that the overall expression of MT1-MMP and furin is reduced in plasma membranes of cells of old normoglycemic animals, a phenomenon that is exacerbated in long-term diabetic animals. This observation supports the prevailing theory that diabetes fosters an acceleration in the aging process. Interestingly, while biochemical results confirmed a decrease in MT1-MMP expression, an increase in furin was observed.

Immunocytochemical studies resolved this discrepancy by tracing the increased furin expression in ER and Golgi membranes of podocytes, indicating that furin might be retained in the secretory pathway in a diabetic environment.

Disturbances at the molecular level of the otherwise tightly regulated MT1-MMP/furin interactions found at the cell surface must account for a lack in extracellular matrix remodeling, increased deposition of GBM material, and loss of glomerular filtration integrity.

Key words: diabetes, glomerulosclerosis, MT1-MMP, furin, GBM turnover

INTRODUCTION

As the ultimate site of plasma filtration, the glomerular wall plays a vital role in maintaining proper permeability for the production of primary urine (1). The integrity of the glomerular wall in terms of structural organization and protein composition is the cornerstone of intact permselectivity. Obvious morphological changes associated with the onset of diabetes and loss of permselectivity include thickening of the glomerular basement membrane (GBM), expansion of the mesangial matrix, and podocyte effacement (2-4). The molecular era of the past two decades has allowed us to witness a wave of information concerning the proteinacious composition of the main filtrating constituents of the glomerular wall, notably the endothelial fenestrations, the GBM, the slit diaphragms at the junction of podocytes, and the mesangial matrix (1,5-6). It has thus recently been possible to identify the molecular mechanisms responsible for progressive glomerulosclerosis at an ultrastructural level.

Electron microscopy employing the immunogold technique as an investigative tool has played an essential role tracing the modifications in molecular composition of glomerular wall components in hyperglycemic environments (7-12). Desjardins et al. in particular, followed the expression and localization of certain key molecular constituents of the GBM and reported that while most molecules remained intact, there were changes in type IV collagen distribution and expression in diabetic rats (12,13). Indeed the importance of collagen deposition and accumulation in the thickening of the GBM cannot be

overemphasized (5,14-15). One of the consequences was a race to identify and locate the enzymes responsible for basement membrane turnover, a process which proves integral to the maintenance of intact glomerular filtration. We then came to realize that the amount and composition of extracellular matrix depends on the delicate interactions existing between synthetic and degradative pathways, and that various forms of glomerular disease are characterized by shifts in this balance (16-17).

The matrix metalloproteases are a large family of zinc endopeptidases widely recognized as crucial mediators in extracellular matrix turnover (18). The membrane type-I metalloprotease distinguishes itself as a membrane-bound enzyme capable of exerting its cleavage activities from the vantage point of the cell surface (19). Osenkwoski et al. underline the regulatory benefits of MT1-MMP's strategic positioning at the membrane. Localization of MT1-MMP at the cell membrane is perfect for pericellular proteolysis allowing for a new set of substrate targets, distinctive interactions with TIMPs and a non-conventional mechanism of regulation involving enzyme internalization, processing and ectodomain shedding (20-21).

The primary function of MT1-MMP is to degrade matrix-associated molecules either directly or via the activation of downstream MMPs (22). Animal knockouts have shown that MT1-MMP is the only MMP known to be essential for survival (20), a fact that is emphasized by its wide array of substrates (22).

The dual role of MT1-MMP as activator of other MMPs capable of initiating activation of zymogen cascades, and as direct cleaver of ECM components, only underscores its vital function as an extracellular matrix remodeler and singles it out as a potential therapeutic target (17,22).

Cleavage of the propeptide region is sine qua non for MT1-MMP activation and subsequent proteolytic activity. Yana and Weiss demonstrated the existence of a proprotein convertase-MT1-MMP axis allowing for the unmasking of MT1-MMP's catalytic domain and conversion into a catalytically active species (23). This was done through the identification of the RX(K/R)R basic motif in the propeptide region of MT1-MMP, a sequence of amino acids that can potentially be recognized by the proprotein convertase family of subtilisin-like proteases. Indeed *in vitro* studies using MT1-MMP deletion mutants confirmed that furin is capable of cleaving the proMT1-MMP zymogen (23). It is thus apparent that cooperative interactions between proprotein convertases and membrane-anchored MMP's play an important role in regulating the remodeling of ECM.

Furin is an endoprotease involved in the proteolytic activation of a large variety of proprotein substrates in secretory pathway compartments (24). The importance of furin as a proprotein convertase capable of processing a wide range of precursors cannot be overemphasized as it has been shown to be ubiquitous in most cell types (25). Specific amino acid motifs identified in furin's cytoplasmic tail have shown to target furin to different cellular compartments; a finding which has shed

light on furin trafficking between the trans-Golgi network, endosomal compartments and the cell surface (26-28). The physiological role of furin in glomerular cells was well characterized by Mayer et al. who co-localized the protein with pro-MT1-MMP in the biosynthetic pathway and traced their translocation beyond the trans-Golgi network onto the cell surface (28). Mayer et al. propose an MT1-MMP activation axis at podocytic and endothelial abluminal membranes facing the GBM for a type of focalized pericellular proteolysis which characterizes the integrated functions of cellular and extracellular elements involved in tissue-remodeling (28). The purpose of our study was to extend this knowledge of interactions among cell surface proteins mediating GBM turnover in normal cells, to their potential modification of expression and/or deregulation in diabetic rat renal tissues.

RESEARCH DESIGN AND METHODS

Antibodies

The rabbit anti-matrix metalloproteinase-14 (MMP-14 or MT1-MMP) was obtained from Sigma-Aldrich (Oakville, ON, Canada). Raised against the N-terminal portion of the enzyme, the antibody tags both mature and pro forms of MT1-MMP. Rabbit anti-furin antibody was supplied by Alexis Biochemicals (Axxora, LLC). The antibody was raised against the N-terminal portion (amino acid sequence 187-198).

Animals

Experimental diabetes was induced in 150g male Sprague-Dawley rats by an intraperitoneal streptozotocin injection (70 mg/kg body weight dissolved in 10 mM/l citrate buffer, pH 4.5). Within 48 hours, animals developed a hyperglycemic state which lasted throughout their lifetime. Glycemia was assessed on a monthly basis with Accu-Check test strips and blood glucose meters (Roche Diagnostics) while glycosuria measurements were carried out weekly using UriScan test strips (YD Diagnostics). Glycemic states at death of 2-month and 12-month old diabetic rats averaged 25.3±4.3 and 27.4±4.0 mmol/L respectively versus 5.6±0.8 mmol/L and 6.7±1.2 mmol/L for their age-matched controls. Four experimental groups were created, each comprising of four animals. Two groups were composed of streptozotocin-injected animals which maintained hyperglycemia for 2 and 12 months, and two were their age-matched

controls. Animals were housed and handled according to the guidelines from the Canadian Council on Animal Care (CCAC).

Glomerular isolation

Animals were anaesthetized with urethane. Upon excision, entire kidneys were immediately recovered in PBS and subjected to sequential sieving methods to separate glomeruli (29). Isolated glomeruli were washed and suspended in freshly-made lysis buffer (29), homogenized, and kept on ice for 1 hour. Homogenates were then centrifuged (2500 rpm) for 20 min at 4° C to remove non-solubilized material. The supernatant carrying membranous proteins MT1-MMP and furin were used for our studies. Protein quantitation was carried out using the bicinchoninic acid method (BCA Protein Concentration Assay, Pierce) with bovine serum albumin as a standard. Samples were aliquoted at concentrations of 40 μg and stored at -80° C until needed.

Immunocytochemistry

Small tissue fragments measuring 1 mm^3 were rapidly sampled from excised kidneys and fixed by immersion in a 4% paraformaldehyde-lysine-periodate solution for two hours at 4°C. Tissue fragments were rinsed in 0.1 mol/l phosphate buffer, dehydrated in methanol and embedded at -30° C in Lowicryl K4M (30). Ultrathin sections were mounted on Parlodion-carbon-coated nickel grids for immunocytochemistry.

MT1-MMP and furin antigenic sites were revealed using the corresponding polyconal antibodies in combination with the protein A-gold complex on a post-embedding immunocytochemical approach as described previously (30). The anti-MT1-MMP was used at 1:50 and anti-furin at 1:10, both overnight at 4°C. Incubation with protein A-gold (10 nm) was carried out for 30 min at room temperature. The tissue sections were stained with uranyl acetate and observed with a Philips 410SL electron microscope. Specificity of immunolabelings was assessed by incubating tissue sections with the protein-A gold complex, omitting the primary antibody step. Competition experiments were also carried out using antigen-adsorbed antibodies.

Precise localization and quantification of both MT1-MMP and furin antigens over glomerular tissue were carried out on electron micrographs with Clemex Vision Analysis unit. Pictures were recorded at X 21 000. Forty pictures were taken for each animal in each experimental group. The glomerular wall was morphologically separated into 7 distinct regions for counting: abluminal and luminal endothelial membranes, abluminal and luminal podocyte membranes, slit diaphragm, mesangial cell plasma membrane, and glomerular basement membrane. In addition, labelings over the rough endoplasmic reticulum and Golgi regions of podocytes, as well as mitochondria were evaluated. The length of these various plasma membrane domains as well as those delineating the cellular compartments were measured and gold particles associated with these membranes counted. Values are expressed in particles per micrometer. Mean

values were calculated along with their standard deviations. Statistical evaluations were carried out using the Student's T-test.

Western blotting

Glomerular protein samples were boiled for 4 minutes in reducing SDS-sample buffer, separated by SDS-PAGE on 10% handmade polyacrylamide gels, and then electrophoretically transferred to nitrocellulose membranes. For MT1-MMP and furin immunodetection, membranes were blocked in TBS (50 mM) containing 0.05% Tween 20 and 1% milk for 1 hour and 3 hours respectively, and then incubated overnight at 4° C with the appropriate antibodies. Antibodies were revealed with Lumi-Light Plus chemiluminescence detection kit and results were exposed with Kodak X-Omat-AR films.

RESULTS

Under examination by electron microscopy, distinct structural features of the renal tissue can be observed. At low magnification it is possible to view the Bowman's capsule surrounding the renal corpuscule composed of endothelial cells facing the capillary lumen and epithelial podocytes facing the urinary space. Mesangial cells are at the junction of podocytes and are embedded within the extracellular matrix while the epithelial podocytic cell bodies project onto the GBM. Most striking observations were podocyte effacement and pronounced thickening of the GBM in tissues of 12-month old hyperglycemic animals and to a lesser degree in tissues of their age-matched normoglycemic animals. No prominent morphological differences were noted between 2-month old animals, normal as well as diabetic.

Immunolabeling

In all tissues, gold particles revealing membrane type-I metalloprotease antigenic sites were found over luminal and abluminal podocyte plasma membranes, in particular at the slit diaphragms, luminal and abluminal endothelial cell membranes (Fig. 1), as well as over mesangial cell plasma membranes. However a specific look at the slit diaphragm region unmistakably revealed a drop in MT1-MMP labeling from tissues of 2-month old control and diabetic rats to tissues of their 12-month counterparts. Gold particles were seen labeling the GBM. Labeling was virtually absent in the urinary space and capillary luminae (Fig. 1).

Morphometrical analyses confirm subjective observations (Table 1). There is an overall decrease in MT1-MMP labeling with age and this decline is most striking in diabetic conditions. Number of gold particles in podocytic abluminal and luminal plasma membranes and endothelial abluminal and luminal membranes of old diabetic animals is significantly less than in young control animals. Overall abluminal and luminal podocyte membrane labelings appear to decrease by little less than half in tissues of old versus young animals. Similarly, there is a steady decline in MT1-MMP labeling in the slit diaphragm regions along with age and diabetes; 2-month old control animals displaying the greatest number and 12-month old diabetic animals exhibiting less than half that number. Labeling in the mesangial cell plasma membranes of young normal and diabetic rats is quite low but increases in old rat tissues, especially in the old diabetic ones. GBM immunolabelings also exhibit a steady increase in MT1-MMP labeling, from young non-diabetic rat tissues to old diabetic ones. Antibody control test measurements revealed no significant labeling (0.014 ± 0.001 particles/μm), confirming the specificity of our results.

Furin immunolabeling was also found over both luminal and abluminal membranes of podocytes and endothelial cells as well as over the mesangial cell plasma membranes (Fig. 2). Labeling of furin in luminal and abluminal endothelial plasma membranes of diabetic rat tissues seemed to be lower than in the non-diabetic ones. There also appeared to be less furin over luminal and abluminal podocytic cell plasma membranes, and in the slit diaphragm regions of

12-month old versus 2-month old rat tissues. Furin in the glomerular wall was in higher concentrations in luminal plasma membranes of podocytes and slit diaphragm junctions, especially in normal rat tissues, while other analyzed regions seemed to be more sparsely decorated. Particles were also found in mesangial cell plasma membranes but they were few in number.

Morphometrical evaluations are in agreement with above observations (Table 2). Gold particles numbered over one particle per micron in 2-month and 12-month old, abluminal and luminal endothelia of control rat and decreased in diabetic age-matched counterparts. Labeling densities over abluminal and luminal podocytic plasma membranes are considerably less in old and diabetic rat tissues when compared to tissues of 2-month old animals. In the slit diaphragms, a two-fold decrease occurred between young normal rats and old diabetic ones while GBM measures oscillate within the same narrow range. Total number of gold particles in mesangial plasma membranes remained low in both normal and diabetic animals. Antibody control testing yield very low values (0.017 ± 0.001 particles/μm) which confirms the specificity of labelings.

Western Blotting

Membranes incubated with anti-MT1-MMP revealed three bands of different molecular weights (Fig. 3). The heaviest band at 63 kDa represents the latent precursor proprotein before activation by cleavage (31). Bands at 57 and 44 kDa were also observed, the former being the active and membranous version of the

protein and the latter representing the inactive ectodomain form (20). Heavy signaling of the 63 kDa latent precursor is seen in young, non-diabetic rat tissue lysates. The intensity of the signal decreases sharply in samples from young diabetic animals and nearly disappears in old diabetic and non-diabetic ones. A similar pattern is observed for the 57 kDa active form of MT1-MMP. In contrast, the 44 kDa band signaling intensity steadily increases from young control to old diabetic animal glomerular lysates, suggesting a marked propensity for autocatalytic degradation of the protein into its inactive form.

Western blotting of glomerular lysates revealed a band at 98 kDa which corresponds to mature rat furin (Fig. 4) (32). Only very faint signaling could be observed in tissues of the 2-month old control and diabetic animals while denser bands appeared in 12-month old control and diabetic rat tissue samples. This steady signaled a discrepancy between electron microscopic observations and biochemical results involving furin. This prompted us to carry on an additional morphological investigation of the intracellular expression of furin.

MT1-MMP and Furin in the ER and Golgi

MT1-MMP immunolabeling was found over ER and Golgi membranes (Fig. 5). There were greater amounts of gold particles in 2-month old normoglycemic and hyperglycemic rat tissues than in their 12-month old counterparts and a quantitative evaluation of these regions reveals a two-fold decrease in labeling (Table 3).

Furin immunolabeling was also found over ER membranes and Golgi cisternae of podocytes (Fig. 6). Differences in labeling were striking between young and old rat tissues and morphometric countings do report a significant increase in furin immunolabeling in 12-month old diabetic and non-diabetic rat tissues in both ER and Golgi, when compared to the young animals (Table 4). In all instances, background labeling measured in neighboring mitochondria remained low for both antigens (Tables 3 and 4).

DISCUSSION

This study looked to further characterize the expression of some key proteins of the glomerular wall associated with the onset of diabetes. The membrane type-I metalloprotease and its activating enzyme furin play a pivotal role in extracellular matrix turnover enticing us to evaluate their expression in diabetic rat renal tissues. By analyzing tissues of short and long term diabetic animals, we found that both enzymes exhibit a decrease in expression in all evaluated plasma membrane regions along with age, an alteration that became more pronounced in diabetic conditions. The only exception was the presence of extracellular MT1-MMP within the glomerular basement membrane which actually increased in old normal and diabetic rat renal tissues. These observations confirm a recurring theme in the study of long-term diabetes, one which proposes that the presence of diabetes accelerates the aging process (11,33-35). The consequences of aging and diabetes are most evidently observed in the thickening of the glomerular basement membrane due to increased deposition and accumulation of proteins that make up the extracellular matrix. In the present study we found two factors which most likely exacerbate GBM thickening: a decrease in MT1-MMP which cleaves a wide array of ECM substrates, concomitant with a decrease in furin, a proprotein convertase which activates MT1-MMP.

With the goal of corroborating immunocytochemical findings, western blots were performed on glomerular lysates. Bands revealed three different forms of MT1-MMP; the pro and active forms significantly diminished in intensity along with

age and diabetes as expected while the truncated inactive form increased in signaling intensity. Furin, on the other hand, gave conflicting results. Instead of a decrease in expression, as found with morphological tools, the 98 kDa furin band increased in intensity in old normal and diabetic rat tissues. These findings prompted us to extend immunocytochemical investigations to the sites of synthesis and processing of MT1-MMP and furin.

The role of furin in pathological situations has been extensively studied (24,36-38), yet less attention has been given to furin in normal physiological conditions. Mayer et al. sought to reverse this trend by localizing endogenous furin in normal renal cells and thus demonstrating its potential interactions with proproteins at the plasma membrane (28,39). Indeed, significant pools of furin and MT1-MMP were seen co-localized and concentrated within the Golgi apparatus of podocytic cells as well as over plasma membranes, suggesting that translocation of pro-MT1-MMP through the secretory pathway and onto the cell surface is facilitated by furin. Once recruited to defined plasma membrane domains of the cell surface, Mayer et al. propose that pro-MT1-MMP is activated by furin which allows for a type of focalized pericellular proteolysis that could be implicated in GBM turnover (28). Our study corroborates Mayer's findings in normal rat renal tissue and goes further to demonstrate the fall in both MT1-MMP and furin expression in diabetic rat tissue. It is important to note, however, that the decrease deals only with cell surface membranes. The possibility that furin could still be residing in

the Golgi and ER membranes enticed us to further our electron microscopy investigations.

Indeed, furin immunolabeling was found concentrated over Golgi and ER cisternae of podocytic cell bodies to a greater extent in old rat renal tissues than in their young counterparts, a trend that was more significant in diabetic conditions. Therefore, there is a possibility that furin, although transcribed and synthesized to a greater degree in a high glucose environment, associates with pro-MT1-MMP in the Golgi but remains trapped in the trafficking pathway. If furin cannot shuttle pro-MT1-MMP to the cell surface, then it cannot activate the metalloprotease for focalized pericellular proteolysis essential to GBM turnover.

Western blot results of MT1-MMP were concomitant with immunocytochemical findings in that the active form of the protein is greatly reduced in diabetes. Interestingly, there is a marked increase in MT1-MMP presence in the GBM of old diabetic rat tissues suggesting a substantial rise in the shed form of the protein. The processes of autocatalysis and shedding of MT1-MMP were thoroughly explored by Toth et al. in an attempt to elucidate the mechanisms of regulation governing MT1-MMP activity at the plasma membrane (21). They observed how ectodomain shedding regulates the pericellular and extracellular activities of MT1-MMP, allowing for either active or inactive truncated versions of the protein to circulate about and act on the extracellular matrix (20-21). This delicate balance in enzyme activity was further layered in complexity as

mechanisms of processing, shedding, homodimerization, and endocytosis of the membrane metalloprotease were discovered (20). Autocatalysis of MT1-MMP at the cell surface is the main mechanism behind the generation of the 44-kDa species, an inactive degradation product lacking the catalytic domain. Osenkowski et al. emphasize how the presence of this inactive version of the protein can compete with the full-length enzyme for collagen binding, reducing collagenolytic activity and cellular invasion of the collagen matrix (20). The negative influence exerted by the 44-kDa species over MT1-MMP enzyme-processing activity may explain how its overabundance in tissues of diabetic animals, as observed in our biochemical findings, still slows down extracellular matrix turnover.

In a study done in 1997, Blanchette et al. brought attention to the activation loop between growth factor TGF- β and furin. With the promoters regulating furin gene expression cloned, it was possible to show in rat synovial cells incubated with TGF- β that this growth factor is capable of coercing furin gene promoters into augmenting furin transcription levels (25). In a previous study, furin had been identified as the converting enzyme responsible for generating mature TGF-β by cleaving the R-H-R-R sequence motif (40). The result is an autofeedback loop mechanism, in which TGF- β upmodulates *fur* gene expression, which in turn increases pro-TGF- β maturation (25). The role of TGF- β in diabetic kidney disease is well documented (16-17,41-43). In cells treated with high glucose concentrations, a significant rise in TGF-β mRNA expression is observed as early

as 24h after the onset of hyperglycemia (16). As a result, the enzyme is capable of instigating factors that promote ECM accumulation such as increasing the synthesis of ECM components while simultaneously downregulating genes that promote matrix degradation (41,44). And so, a potential new link between furin and diabetes is made. If the expression of TGF- β is upregulated in diabetic conditions, then active TGF- β in turn increases the transcription of its own activating enzyme furin. Although furin is overexpressed in a hyperglycemic environment, as our biochemical results suggest, analysis by electron microscopy leads us to believe that the enzyme is retained in the ER and Golgi compartments of the protein-trafficking pathway. This prevents it from reaching the cell surface where cleavage of metalloproteases such as MT1-MMP can occur for GBM maintenance and turnover.

In 1994, Nakamura et al. pointed out that the expression of many metalloproteases was downregulated in streptozotocin-induced diabetic rats (45). The abnormal gene regulation of MT1-MMP in particular was eventually linked to TGF- β. In high glucose conditions, TGF- β expression is increased and may alter MT1-MMP promoter activity through inhibition (46). Defects at the MT1-MMP gene transcription level in diabetic conditions thus accounts for the loss of its proteolytic activities in GBM turnover as inactive species accumulate and active membrane species are not replenished. The decrease in MT1-MMP expression in diabetes has been widely reported in the literature (16-17,42,47) and was also

observed by electron microscopy at the level of plasma membranes and Golgi compartments of glomerular cells in our study.

TGF- β thus reveals itself as a double-edged sword; on the one hand it upregulates furin gene transcription and on the other it downregulates MT1-MMP gene transcription through indirect interactions with their promoter regions. An upregulation in furin synthesis by TGF- β does not seem to compensate for TGF- β downregulation of GBM proteolytic proteins such as MT1-MMP, and upregulation of ECM component synthesis like collagen; factors that contribute to the thickening of the basement membrane as seen in glomerulosclerosis. As a result, not only does furin have less of its substrate to cleave, but our electron microscopy results seem to suggest that the proprotein convertase is retained in the biosynthetic pathway. To substantiate this proposition, there is a need for further studies involving other furin substrates that are also membrane-bound but not downregulated in diabetes. If expression of these proteins at the cell surface is reduced, then evidence for furin retention in the secretory pathway could be further strengthened.

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TABLE 1

Quantitative Evaluation of Immunogold Labeling for Membrane Type-I Metalloprotease over the Glomerulus

	Endothelial Cells		Podocytes			Mesangial Cells	Glomerular Basement Membrane
Animals	Abluminal Membrane	Luminal Membrane	Abluminal Membrane	Luminal Membrane	Slit Diaphragm	Plasma Membrane	
2 month, control	0.68 ± 0.14[a]	0.91 ± 0.19	1.11 ± 0.20	1.87 ± 0.23	1.29 ± 0.25	0.22 ± 0.08	0.87 ± 0.14
2 month, diabetic	0.62 ± 0.16	0.77 ± 0.22	0.84 ± 0.17	1.06 ± 0.16	0.99 ± 0.17	0.23 ± 0.08	1.17 ± 0.15
12 month, control	0.56 ± 0.11	0.55 ± 0.16	0.54 ± 0.13*	0.82 ± 0.17	0.53 ± 0.14*	0.52 ± 0.12*	1.13 ± 0.17
12 month, diabetic	0.48 ± 0.16	0.79 ± 0.22	0.46 ± 0.15*	0.74 ± 0.16	0.49 ± 0.19*	1.14 ± 0.18*	1.44 ± 0.18

[a] Gold particles per μm, mean values ± SEM.

*$p < 0.05$ (Each different condition was compared to the 2-month control animal group)

TABLE 2

Quantitative Evaluation of Immunogold Labeling for Furin over the Glomerulus

	Endothelial Cells		Podocytes			Mesangial Cells	Glomerular Basement Membrane
Animals	Abluminal Membrane	Luminal Membrane	Abluminal Membrane	Luminal Membrane	Slit Diaphragm	Plasma Membrane	
2 month, control	1.05±0.14 [a]	1.38±0.15	1.11±0.16	1.90±0.21	1.33±0.18	0.20±0.06	0.59±0.08
2 month, diabetic	0.83±0.13	0.72±0.12	0.64±0.12	0.95±0.15*	0.99±0.19	0.21±0.04	0.45±0.09
12 month, control	1.15±0.22	1.22±0.34	0.60±0.16*	0.97±0.19*	0.76±0.17	0.34±0.05	0.46±0.07
12 month, diabetic	0.54±0.11*	0.63±0.13*	0.57±0.11*	0.99±0.18*	0.64±0.14*	0.34±0.08	0.42±0.07

[a] Gold particles per μm,, mean values ± SEM.

*p< 0.05 (Each different condition was compared to the 2-month control animal group)

TABLE 3

Quantitative Evaluation of Immunogold Labeling for Membrane Type-I Metalloprotease over Podocytic Cellular Compartments

Animals	Endoplasmic Reticulum	Golgi Apparatus	Mitochondria
2 month, control	0.85±0.06 [a]	0.89±0.09	0.15±0.09
2 month, diabetic	0.77±0.05	0.86±0.1	0.16±0.12
12 month, control	0.44±0.04	0.45±0.1	0.19±0.03
12 month, diabetic	0.42±0.04	0.55±0.08	0.18±0.02

[a] Gold particles per μm, mean values ± SEM.

TABLE 4

Quantitative Evaluation of Immunogold Labeling for Furin over Podocytic Cellular Compartments

Animals	Endoplasmic Reticulum	Golgi Apparatus	Mitochondria
2 month, control	0.59±0.05 [a]	0.43±0.07	0.15±0.05
2 month, diabetic	0.55±0.06	0.46±0.07	0.12±0.06
12 month, control	0.85±0.08	0.64±0.07	0.20±0.05
12 month, diabetic	0.86±0.08	0.66±0.08	0.19±0.04

[a] Gold particles per μm,, mean values ± SEM.

LEGENDS

FIG.1: Electron micrographs of (A) short-term control and (B) long-term diabetic rat renal tissues. Colloidal gold labeling demonstrates presence of MT1-MMP in the glomerular wall. There seems to be a significant decrease in labeling in tissues of diabetic animals. Thickening of the glomerular basement membrane (GBM) in the tissues of the diabetic animal (B) is evident. US= urinary space, P= podocyte, CL= capillary lumen. Bars = 0.25 μm.

FIG.2: Electron micrographs of (A) short-term control and (B) long-term diabetic rat renal tissues. Colloidal gold labeling of furin reveals less labeling in the glomerular wall of the diabetic animal. US= urinary space, P= podocyte, CL= capillary lumen. Bars = 0.25 μm.

FIG.3: Western blot analysis of MT1-MMP in glomerular lysates: (a) 2 month control, (b) 2 month diabetic, (c) 12 month control, (d) 12 month diabetic. Varying band intensities were found at three molecular weights: the 63 kDa pro-form, the 57 kDa active form and the 44 kDa inactive form.

FIG.4: Western blot analysis of furin in glomerular lysates: (a) 2 month control, (b) 2 month diabetic, (c) 12 month control, (d) 12 month diabetic. The furin band can be seen at 98 kDa.

FIG.5: Electron micrograph of 2 month control rat renal tissue Golgi region of a podocyte. Gold particles labeling MT1-MMP are found in rough endoplasmic reticulum (RER) and Golgi (G) cisternae. Bar = 0.25 μm.

FIG.6: Electron micrographs of furin immunolabeling. Pericytes from (A) young control and (B) old diabetic animals. A and B illustrate rough endoplasmic reticulum (RER) cisternae. The labeling is drastically increased in tissues of diabetic animals. Pericytes from (C) young control and (D) old diabetic animals. C and D illustrate the Golgi apparatus (G). Again labeling is increased in tissues of diabetic animals (D). Bars = 0.25 μm.

Figure 1

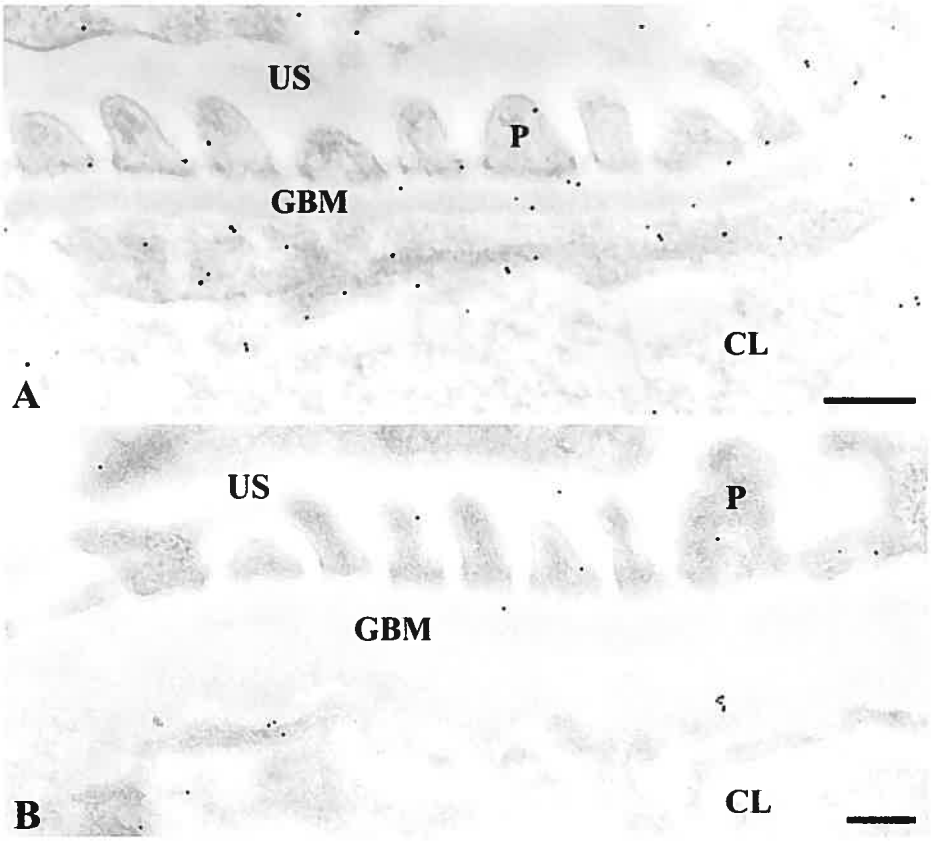
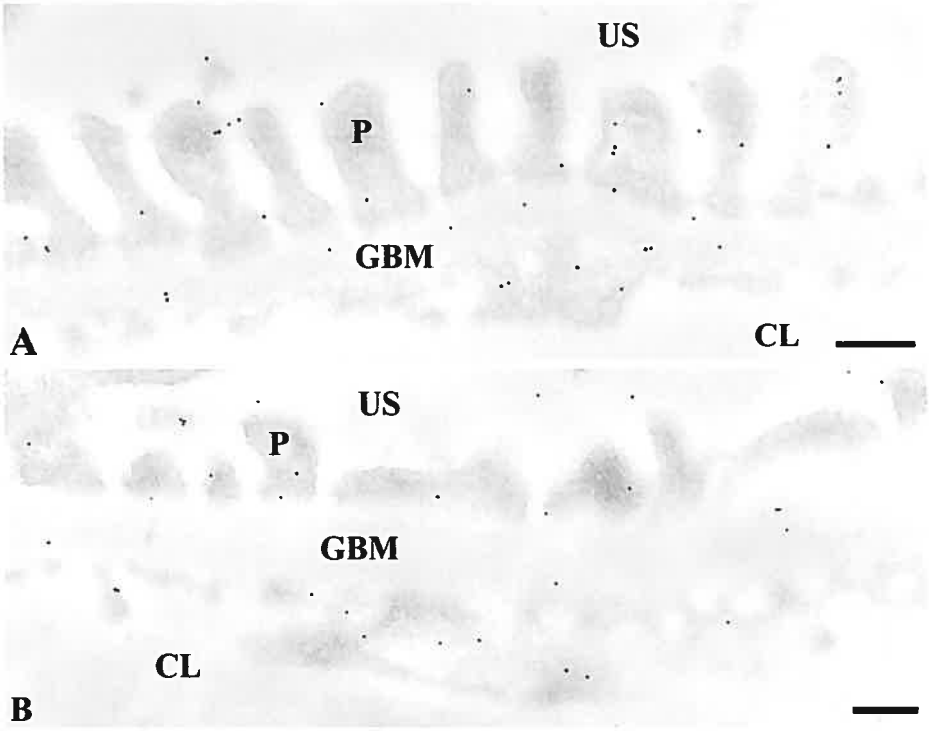


Figure 2



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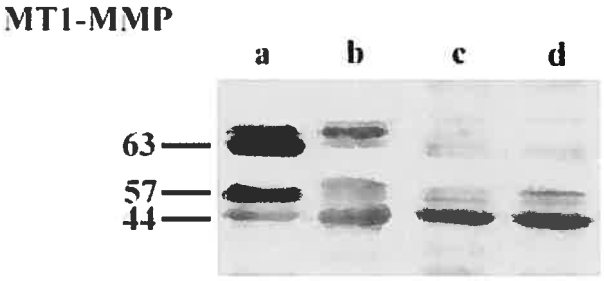

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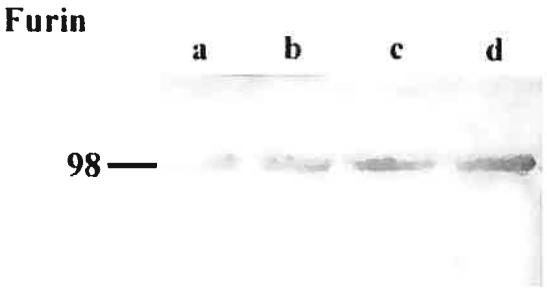


Figure 5

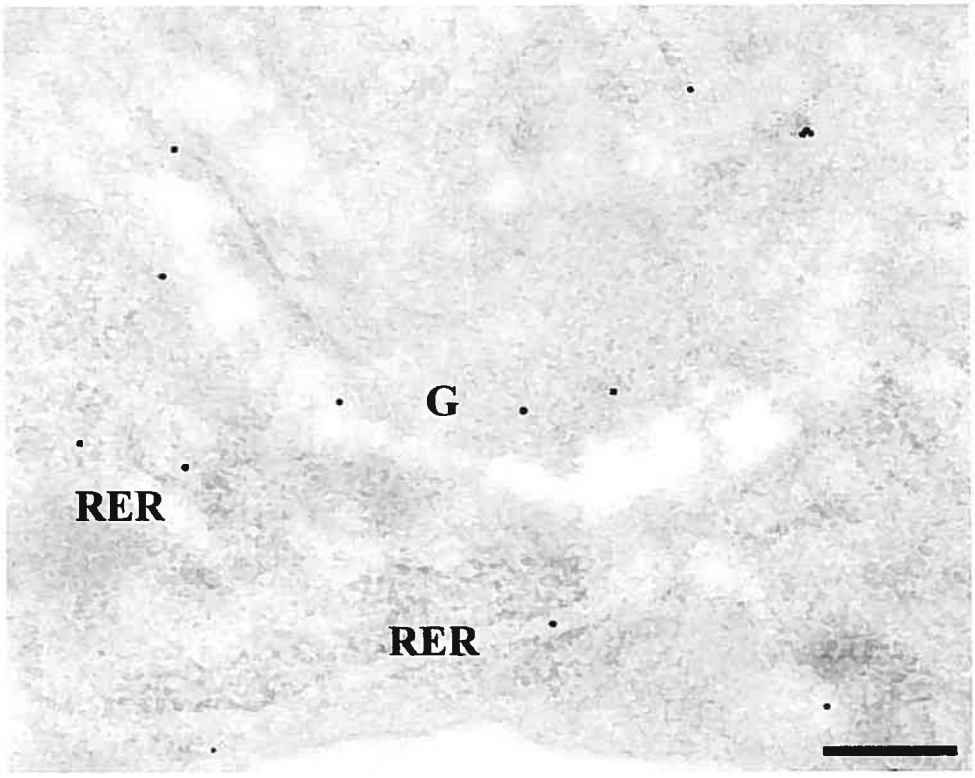
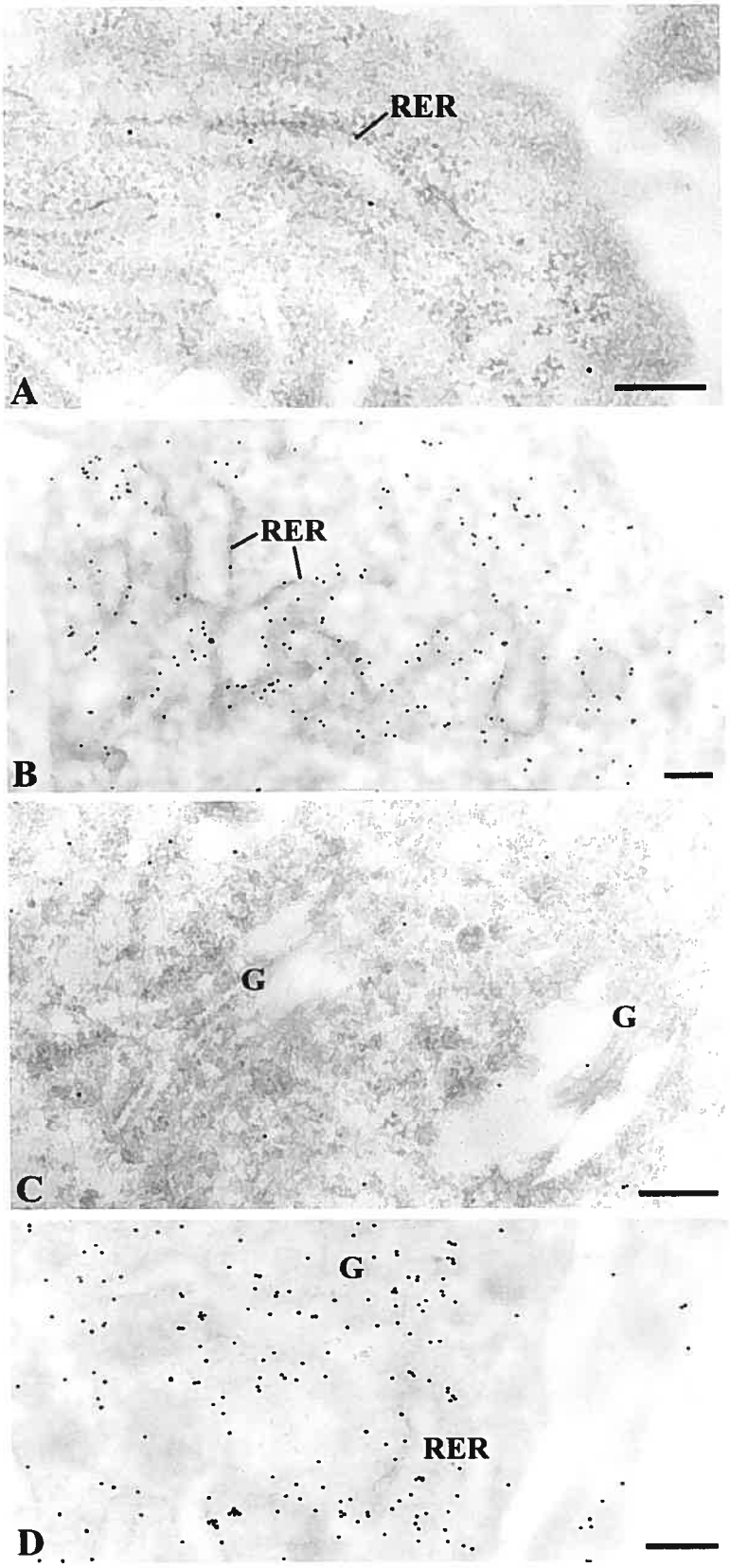


Figure 6



Discussion

With the advent of increasingly refined morphological tools, we have sought to shed additional light on the abnormal loss of proteins in the urinary space at an ultrastructural level. It is now clear that generalized proteinuria is largely a result of alterations in the selective filtration properties of the GBM, and that these functional changes occur earlier than observable structural modifications (Doucet et al., 1999). Nevertheless, that the thickening of the glomerular basement membrane reflects biochemical modifications in its amino acid and sugar composition, and that this aggravates proteinuria, has been known for quite some time (Spiro and Spiro, 1968; Beisswenger and Spiro, 1970; 1973; Beisswenger, 1976; Spiro, 1976), and due to its complexity is still a hot topic among histochemical circles (Kanwar, 1984; Kim et al., 1991; Desjardins and Bendayan, 1990; Inoue and Bendayan, 1995; McCarthy, 1997; Regoli et al., 1998). Loss of glomerular function in diabetes in particular, has been linked with modifications in the selective properties of the glomerular wall (Deckert et al., 1988). Our project sought to deepen our knowledge on the morphological and biochemical changes that accompany basement membrane thickening in diabetes in order to further clarify the events that lead to glomerular dysfunction.

The recent molecular era has exponentially increased our knowledge of the biochemical modifications that accompany basement membrane thickening. Of

interest are the specific molecular interactions orchestrating basement membrane turnover and maintenance. The matrix metalloproteases are a family of endoproteases that were identified as key players in extracellular matrix turnover. Our increased awareness of MMPs is mostly a result of their relevance in cancer biology, namely cancer-cell growth, differentiation, apoptosis, migration and invasion, yet we are increasingly researching their mechanisms of action in other pathological processes such as diabetes. When Yana and Weiss (2000) demonstrated the existence of a furin cleavage site in the membrane type I matrix metalloprotease, it became clear that cooperative interactions between proprotein convertases and membrane-anchored MMP's play an important role in regulating the remodeling of the extracellular matrix. With respect to glomerular filter integrity, it also became clear that proper permselectivity relies on the delicate interactions existing between synthetic and degradative pathways, and that various forms of glomerular diseases are characterized by shifts in this balance (Del Prete et al., 1998; Lenz et al., 2000; Stamenkovic, 2003).

In the present study, the expression of membrane type-I matrix metalloprotease and furin, two enzymes known to be crucial in extracellular matrix remodeling, was evaluated in the glomerular wall of diabetic rat renal tissues. This was done with experimentally-induced diabetic rats by streptozotocin injection. Streptozotocin selectively destroys pancreatic beta-cells and requires only one dose to induce an hyperglycemic state in animals wich occurs within 48 hours, a condition that remains throughout their lifetime. In this study, hyperglycemic

states were maintained for 2 months (short-term) and 12 months (long-term) before sacrifice. Male Sprague-Dawley rats were used because they systematically develop symptoms and alterations that are characteristic of diabetic glomerulopathies (Steffes and Mauer, 1984). Given that a small number of beta pancreatic cells survive streptozotocin treatment, injected rats are still capable of producing small amounts of insulin. This means they will perpetually maintain an hyperglycemic state without requiring a constant supply of additional insulin. Before sacrificing animals for study, the weight, glycosuria and glycemic levels were measured. Pathological conditions reflecting diabetes could thus be evaluated. Indeed, glycemic levels and glycosuria were significantly higher in rats injected with streptozotocin than normoglycemic animals, and corporal weight significantly lower in those same diabetic animals.

For immunocytochemical techniques, the post-embedding technique was employed. Colloidal protein A-gold was used to reveal the desired antigenic sites; this method is useful for the high resolution it offers and the ease with which morphometrical analyses can be carried out to determine its distribution in the glomerular wall.

At high magnification under electron microscopy, it is possible to view the distinct cellular structures that make up the glomerular wall. The endothelial cells are facing the capillary lumen and the epithelial podocytes face the urinary space. Mesangial cells are at the junction of podocytes and are embedded within the

extracellular matrix while the epithelial podocytic cell bodies project onto the GBM. Significant thickening of the GBM is only apparent in older normoglycemic and hyperglycemic rat renal tissues. In 2-month old tissues, GBM width is thinner and does not differ significantly between normal and diabetic conditions. In addition, an important accumulation in mesangial extracellular matrix can be observed in long-term diabetic tissues.

These observations confirm a recurring theme in the study of long-term diabetes, one which proposes that the presence of diabetes accelerates the aging process (Darmady et al., 1973; Bolton et al., 1976; Quagliano et al., 1993; Bendayan, 1998). For example, advanced glycated end (AGE) products were detected in the renal extracellular matrix of old normoglycemic animals as well as younger diabetic tissues, indicating that AGE products may participate in the pathogenesis of renal complications (Bendayan, 1998). Indeed, in several instances spontaneous glomerulosclerosis was observed in aging normoglycemic rat tissues (Bolton et al., 1976; Quagliano et al., 1993). However, in instances where chronic glycemia prevails, the process of glomeruloscelrosis is significantly accelerated in younger animals (Couchman et al., 1994; Lee et al., 1997). The present study also noted significant GBM thickening due to increased deposition and accumulation of proteins that make up the glomerular wall of both old normoglycemic and hyperglycemic rat renal tissues. Most importantly, our results bring to attention two factors which most likely exacerbate this GBM thickening: a decrease in MT1-MMP which cleaves a wide array of ECM

substrates, concomitant with a decrease in furin, a proprotein convertase which activates MT1-MMP.

Immunogold labeling consistently revealed the presence of MT1-MMP and furin enzymes over the apical and basal plasma membranes of epithelial cells, the slit diaphragm region, and the luminal and abluminal endothelial membranes. General qualitative observations revealed that immunolabeling for both enzymes was especially frequent in the luminal and abluminal membranes of podocytes, as well as the slit diaphragms of young normal rat renal tissues. This is in accordance with Mayer et al.'s findings on the co-localization of these proteins in those same areas (Mayer et al., 2003). Moreover, Mayer et al. noted that significant pools of furin and MT1-MMP were seen co-localized and concentrated within the Golgi apparatus and intracellular vacuoles of podocytic cells as well as over plasma membranes, suggesting that translocation of pro-MT1-MMP through the secretory pathway and onto the cell surface is facilitated by furin. Furin/pro-MT1-MMP complexes were also present at the slit diaphragm domain, at the abluminal side of endothelial cells facing the GBM and in the vicinity of endothelial fenestrations (Mayer et al., 2003). Once recruited to defined plasma membrane domains of the cell surface, it is proposed that pro-MT1-MMP is activated by furin which allows for a type of focalized pericellular proteolysis that could be implicated in GBM turnover (Mayer et al., 2003). Indeed, it has been suggested that anchoring furin at the plasma membrane might provide a mechanism for concentrating the protease in discrete regions where efficient

extracellular processing could take place (Liu et al., 1997). Our observations of MT1-MMP and furin immunolabeling over those same regions of the glomerular wall of normal rat renal tissues provide additional support to Mayer et al.'s proposition of a focalized pericellular proteolysis at the surface of cells.

The next step was to look at the expression and localization of MT1-MMP and furin in the glomerular wall of rat renal tissues, but in hyperglycemic conditions. We hypothesized that any modifications in expression at this level may signal perturbations in focalized pericellular proteolysis normally carried out by these enzymes for GBM turnover. Through morphometrical analysis of tissues of short- and long- term diabetic animals, we found that both enzymes exhibit a decrease in expression in all evaluated plasma membrane regions along with age, an alteration that became more pronounced in the diabetic condition. The only exception was the presence of extracellular MT1-MMP within the glomerular basement membrane which actually increased in old normal and diabetic rat renal tissues. Interestingly, there was a two-fold decrease in immunolabeling specifically within the slit diaphragm regions of old diabetic tissue versus young normal tissues. This observation lends additional credence to Mayer et al.'s theory of focalized pericullar proteolysis in specific plasma membrane microdomains.

With the goal of corroborating immunocytochemical findings, western blots were performed on glomerular lysates. Bands revealed three different forms of MT1-

MMP; the pro (63 kDa) and active (57 kDa) forms which, as expected, significantly diminished in intensity along with age and diabetes. Interestingly, the truncated inactive form observed at 44 kDa actually *increased* in signaling intensity. As reported by Osenkowski et al., the 44 kDa species is the inactive degradation product of MT1-MMP proteloytic processing at the cell surface, which translates into a potential regulation mechanism of the protein (Osenkowski et al., 2004).

The process of ectodomain shedding is well established as a major regulatory mechanism that controls the activity of a variety of membrane-bound proteins on the cell surface. Ectodomain shedding of MT1-MMP was thoroughly explored by Toth et al. who suggest that this mechanism evolved as a way to terminate MT1-MMP activity at the cell surface (Toth et al., 2002). In this unique regulatory mechanism, active MT1-MMP undergoes autocatalytic processing on the cell surface which leads to the formation of an inactive 44 kDa species and release of the entire catalytic domain (Toth et al., 2002). By disrupting enzyme integrity at a vital structural site, ectodomain shedding regulates the pericelluar and extracellular activities of MT1-MMP through a delicate balance of active and inactive enzyme-soluble fragments. This process either switches the proteolytic machinery from the cell surface to the pericellular space if the released fragments are competent enzymes or may obliterate proteolysis, if the soluble fragments are inactive and/or in a complex with TIMPs (Toth et al., 2002). The loss of the catalytic domain clearly terminates MT1-MMP activity on the cell surface but the

remaining 44 kDa degradation product can also negatively influence enzymatic activity. The 44 kDa species was shown to compete with the full-length enzyme for collagen binding, reducing collagenolytic activity (Tam et al., 2002). The negative influence exerted by the 44-kDa species over MT1-MMP pericellular processing activity may explain how its overabundance in tissues of diabetic animals, as observed in our biochemical findings, still slows down extracellular matrix turnover. In contrast, inhibition of this shedding has been shown to lead to an accumulation of the active enzyme on the cell surface and so an enhancement in MT1-MMP proteolytic activity (Toth et al., 2002). In conclusion, Toth et al. remark that the ability of cells to elicit autocatalytic shedding depends on the expression level of MT1-MMP on the cell-surface and the levels and availability of TIMPs. In the case of diabetes, our results suggest that MT1-MMP undergoes autocalytic processing at the membrane, yet newly synthesized forms of the protein are not replenished by the cell. The result is an accumulation of the inactive degradation product in the extracellular matrix, leading to a reduction in pericellular proteolysis, which aggravates basement membrane thickening.

Osenkowski et al. point out that the total amount of MT1-MMP on the cell surface depends on the balance between autocatalytic processing, internalization, and *de novo* protein synthesis (Osenkwoski et al., 2004). The effect of hyperglycemia on MT1-MMP autocatalysis and shedding still remains cloudy, yet studies documenting the influence of a high glucose environment on MT1-MMP gene expression and synthesis are numerous (Nakamura et al., 1994; Lenz et al.,

2000; McLennan et al., 2000; McLennan et al., 2002; Portik-Dobos et al., 2002; Dolan et al., 2003; Mason and Wahab, 2003).

In 1994, Nakamura et al. observed that levels of mRNA encoding for many metalloproteases was downregulated in streptozotocin-induced diabetic rats (Nakamura et al., 1994). A new link between hyperglycemia and abnormal gene regulation of MMPs was made. Subsequent studies on MT1-MMP expression in a diabetic environment reported similar observations. MT1-MMP expression and total MMP activity were decreased by two-fold in the arterial vasculature of diabetic patients (Portik-Dobos et al., 2002). With respect to glomerular cells, McLennan et al. observed that high glucose concentrations can decrease degradation of mesangium by reducing the activities of MT1-MMP and other MMPs (McLennan et al., 2002). Mason and Wahab extensively reviewed the effect of diabetic nephropathy on extracellular matrix metabolism and charted the increased and decreased activities of MMPs (Mason and Wahab, 2003). MMP-7, MMP-9 and MT1-MMP mRNA diminish in expression in mesangial cells while MMP-2 expression is augmented but activation seems to be reduced (McLennan et al., 2000). The decrease in MT1-MMP expression in diabetes has thus been well established in the mesangial matrix, suggesting a decrease in turnover and therefore excessive matrix accumulation. Our study observed a similar pattern by electron microscopy at the level of plasma membranes of epithelial and endothelial cells of the glomerular wall. When we extended our immunocytochemical investigations to the biosynthetic pathways of podocytic

cells, the putative sites of MT1-MMP synthesis, we noticed a drop in innmunolabeling at the ER and Golgi cisternae of old diabetic rat renal tissues versus young normal ones. These observations reconfirmed the drop in mRNA expression of MT1-MMP in a high glucose environment, a fact also supported by our biochemical findings.

The abnormal gene regulation of MT1-MMP was eventually linked to growth factor TGF- β. To date, three TGF- β isoforms have been identified (TGF- β1, β2, and β3) in the mammalian species. The TGF- βs are a family of ubiquitously expressed cytokines which have attracted much attention due to their pleitropic biological activities (Blanchette et al., 1997). Biological processes in which TGF-β involvement is apparent include embryogenesis, cell cycle arrest, wound healing, cell differention, tissue fibrosis, and most importantly with respect to our study, increased synthesis of exracellular matrix components (McCartney-Francis and Wahl, 1994).

Expression of TGF- β in glomerular cells is weak in the normal state. It is now well-documented that in high glucose conditions, the expression of all three TGF-β isoforms is increased (Dolan et al., 2003). There is extensive evidence for the pathological role of TGF- β in diabetic nephropathy. TGF- β induces the production of extracellular matrix components such as type-IV collagen and fibronectin (Marti et al., 1994). It also inhibits matrix turnover by down-regulating matrix metalloproteases and stimulating TIMPs (Dolan et al., 2003;

McLennan et al., 2000). TGF- β thus doubly contributes in the increased production of extracellular matrix in diabetes: first by up-regulating synthesis of basement membrane components and second, by down-regulating synthesis of endoproteases which cleave these components for matrix breakdown. TGF- β has indeed been demonstrated to interfere indirectly with the promoter regions of MT1-MMP (McLennan et al., 2000). Defects at the MT1-MMP gene transcription level in diabetic conditions is in accordance with our immunocytochemical and biochemical findings which suggest that inactive MT1-MMP species accumulate in the glomerular basement membrane while active membrane species are not replenished at the surface from the biosynthetic pathway.

Furin, on the other hand, gave conflicting results. Instead of a decrease in expression, as seen in the glomerular walls of old normal and diabetic animals by electron microscopy, the 98 kDa furin band actually increased in intensity in old normal and diabetic rat tissues. Unsatisfied with the discrepancies obtained between electron microscopy and biochemical results, we looked to further our immunocytochemical investigations to the ER and Golgi membranes of podocytes, also the putative sites of synthesis of the furin protease. Indeed, furin immunolabeling was found concentrated over Golgi and ER cisternae of podocytic cell bodies to a greater extent in old rat renal tissues than in their young counterparts, a trend that was more significant in diabetic conditions. Therefore, there is a possibility that furin, although transcribed and synthesized to a greater

degree in a high glucose environment, associates with pro-MT1-MMP in the Golgi but remains trapped in the trafficking pathway. If furin cannot shuttle pro-MT1-MMP to the cell surface, then it cannot activate the metalloprotease for focalized pericellular proteolysis essential to GBM turnover.

In 1997, Blanchette et al. released a paper linking furin expression with none other than growth factor TGF-β. Their study brought to light an activation loop existing between growth factor TGF-β and furin (Blanchette et al., 1997). In a previous study, furin had been identified as the converting enzyme responsible for generating mature TGF-β by cleaving the R-H-R-R sequence motif (Dubois et al., 1995). The promoters regulating furin gene (*fur*) having been cloned (Ayoubi et al., 1994), Blanchette et al. were able to demonstrate that TGF-β is capable of coercing *fur* gene promoters into augmenting furin transcription levels. The result is an auto-feedback loop mechanism, in which TGF-β upmodulates *fur* gene expression, which in turn increases pro-TGF-β maturation (Blanchette et al., 1997). As we have seen, the role of TGF-β in diabetic kidney disease is well documented (Dolan et al., 2003; Lenz et al., 2000; Del Prete et al., 1998; Portik-Dobos et al., 2002). In cells treated with high glucose concentrations, a significant rise in TGF-β mRNA expression is observed as early as 24h after the onset of hyperglycemia (Dolan et al., 2003). And so, a potential new link between furin and diabetes is made. If the expression of TGF-β is upregulated in diabetic conditions, then active TGF-β in turn increases the transcription of its own activating enzyme furin. Although furin is overexpressed in a hyperglycemic

environment as our biochemical results suggest, analysis by electron microscopy leads us to believe that the enzyme is retained in the ER and Golgi compartments of the protein-trafficking pathway. This would prevent it from reaching the cell surface where cleavage of metalloproteases such as MT1-MMP can occur for GBM maintenance and turnover.

Conclusion

The expression and localization of enzymes essential to glomerular basement membrane maintenance and renewal was evaluated in the renal tissues of normal and diabetic rats. By employing immunocytochemical techniques, we observed a significant drop in the expression of both MT1-MMP and furin in selected membrane domains of the glomerular wall of animals exposed to a hyperglycemic environment. There thus appears to be alterations at the level of proteins mediating basement membrane turnover at the glomerular cell plasma membranes of diabetic animals. Interestingly, there was a two-fold decrease in expression of both proteins specifically in the slit diaphragm regions at the junction of podocytes and GBM. That MT1-MMP and furin activity is concentrated in the slit diaphragm regions was an idea advanced by Mayer et al. (2003) who detected frequent co-localization of these proteins in those specific areas. Our results suggest that the proposed mechanism of focalized pericellular proteolysis mediated by MT1-MMP and furin at the cell surface of podocytes is disrupted in diabetic conditions.

While western blots confirmed a diminution in expression in the active form of MT1-MMP, the bands representing the inactive shed form of the protein appeared to increase in intensity in old diabetic animals. Indeed, immunolabeling of shed forms of MT1-MMP in the glomerular basement membranes of diabetic animals

was greater than that of normal animals, indicating an increase in the autocatalysis of the endoprotease at the cell surface of podocytes.

Biochemical results for furin, on the other hand, gave contrary results to what was expected. Instead of decreasing in signaling intensity, the bands representing furin increased with respect to age, and were the most intense in old diabetic animals. In light of these biochemical observations, the possibility was raised that furin may be residing elsewhere than the cell surfaces of glomerular cells. Immunocytochemical investigations were thus extended to the biosynthetic pathway of podocytic cell bodies where furin is believed to shuttle proteins towards the cell surface. Additional quantitative evaluations of furin labeling in the ER and Golgi cisternae indeed signaled the increased presence and retention of furin in those membranes under diabetic conditions.

It was found in the literature that the furin gene contains a promoter region that can be activated by TGF-β (Blanchette et al., 1997). The increased expression of TGF-β has been demonstrated in hyperglycemic conditions (Dolan et al., 2003; Lenz et al., 2000), and in light of this study a potential new link between furin, TGF-β, and diabetes is made. While augmented levels of TGF-β in high glucose conditions act positively on the promoter region of furin to increase its expression, the protease does not reach the cell surface due to its retention in the biosynthetic pathway of podocytes and so cannot perform its duty as activator of basement membrane endoproteases such as MT1-MMP. Furthemore, TGF-β has

already been shown to downregulate MMP expression (McLennan et al., 2000) and upregulate the synthesis of ECM components (Marti et al., 1994), two factors which only contribute further to the thickening of the glomerular basement membrane.

To verify that furin is indeed blocked in the biosynthetic pathway of podocytes, there is a need for further studies involving other furin substrates that are also membrane-bound but not downregulated in diabetes. Therefore, if expression of these proteins at the cell surface is reduced, then this would provide additional evidence that the retention of furin in the secretory pathway impinges on proper localization and function of furin and its substrate for pericellular proteolysis at cell surface membranes. This would highlight a new pathological role for furin in diabetes and offer insights into potential therapeutic targets for reducing basement membrane thickening in diabetic glomerulosclerosis.

Finally, the similar alterations in morphological charactersistics and protein expression observed in 12-month old normal and diabetic tissues provides additional weight to the theory that diabetes fosters an acceleration in the aging process. Changes that thus occur under normal circumstances in the glomerular wall with age, i.e. podocyte effacement as well as basement membrane thickening and mesangial matrix expansion, are thus significantly enhanced in a hyperglycemic environment. Our study further supports this by demonstrating the decreased expression of MT1-MMP and furin in both normal and diabetic 12-

month old rat renal tissues, a factor which probably correlates with the basement membrane thickening observed in those older animals.

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